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THE ASSAY OF ANDROSTERONE BY THE CHICK COMB WEIGHT METHOD AND THE RENAL EXCRETION OF ANDROGENS BY THE CEBUS MONKEY

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THE GRADED response to androsterone and other androgenic material by the comb method was studied by Frank, Klempner, Hollander and Kriss (1942) using baby chicks instead of adult capons (Gallagher and Koch, 1935; Greenwood, Blyth and Callow, 1935). Advantages of employing chicks are quite obvious: it is easier to use large numbers for each dose, to have more uniform groups within the laboratory and, most important, to assay small amounts of hormone. The empirical equation given by the New York investigators, however, was justifiably criticized by Bliss and Cattell (1943). This work was done to get more information about the reliability of the baby chick as a test animal for androgens and to assay urinary extracts of South American monkeys of the genus *Cebus* according to the method and recommendations of Bliss and Marks (1939).

MATERIAL AND METHODS

White, male, 3 day old Leghorn chicks obtained from the same hatchery were used. Batches of 100 birds shipped to the laboratory the second day after hatching were received 8 hours later. They were placed in 4 floor brooders, 20 animals in each floor, at 32°–37°C. and given bread and milk *ad libitum* plus the following diet: corn meal 50%, rice bran 20%, wheat bran 15%, meat meal 10% and bone meal 5%.

A sample of androsterone (m.p. 182°–184°C.) dissolved in peanut oil was used throughout the experiments. The different groups and doses appear in Table 1.

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The samples of urine were collected under toluol from 6 adult male cebus monkeys weighing 2.5–4.5 kg. The collection ran through periods of 24 hours except for 30 minute intervals, 3 times a day, when the animals received drinking water and food. The daily samples of urine with a pH not higher than 6.5 were pooled, acidified with 1% HCl and placed in the ice-box. Details for hydrolysis, extraction and fractionation have been given (Henriques and Henriques, 1946).

TABLE 1. SUMMARY OF STATISTICS FOR RELATION BETWEEN WEIGHT OF THE COMB OF MALE CHICKS AND THE DOSAGE OF ANDROSTERONE

Assay #	Dose μg.	Log. of the dose	Mean weight of the comb (mg.)	Variance	Number of chicks	Slope	Sigma of the slope
I	15.0	1.176	46.7	267.95	20		
	22.4	1.350	53.1	272.77	20		
	33.5	1.525	62.0	699.82	19	42.264	11.52
	50.0	1.699	68.3	408.86	18		
II	15.0	1.176	41.9	247.62	18		
	22.4	1.350	49.0	218.30	14		
	33.5	1.525	56.3	261.76	16	41.685	10.56
	50.0	1.699	63.7	652.37	16		
III	15.0	1.176	55.2	305.41	14		
	22.4	1.350	60.9	389.34	16		
	33.5	1.525	67.3	360.17	16	41.516	11.42
	50.0	1.699	77.2	599.61	12		
IV	15.0	1.176	42.5	204.34	17		
	22.4	1.350	48.9	253.78	20		
	33.5	1.525	64.0	384.08	14	55.975	10.04
	50.0	1.699	70.0	403.50	18		
V	75.0	1.875	91.4	771.28	20		
	112.0	2.049	110.3	689.51	19		
VI	50.0	1.699	78.8	606.74	17		
	75.0	1.875	91.8	797.63	16		
	112.0	2.049	118.1	949.14	18	115.114	16.14
	168.0	2.225	136.1	794.86	16		

The oil solution of the hormone or of the extract, 0.05 cc. once a day for 7 successive days, was applied without inunction directly to the comb of the baby chicks. Twenty four hours after the last dose the birds were killed, the sex determined and the comb dissected and weighed as described by Frank and coworkers (1942). The males that did not present a body weight increase of at least 2 g. and all females were discarded.

The comparative assay was performed after checking the linearity of the response to 3.5, 7.0 and 14.0 μg. of androsterone. The final assay of the urinary extract was made by using 4 groups of 11 chicks and the doses of 7 and 14 μg. of androsterone (S_1 and S_2) and the amount of the extract corresponding to 30 and 60 cc. of urine (U_1 and U_2).

RESULTS

Average weight of the comb of the baby chick according to the

different experimental groups and the dosage of androsterone used are presented in Table 1. A linear relationship exists between the response and the logarithm of the dose although the position of the straight lines varied from one assay to another (fig. 1). Also the slopes

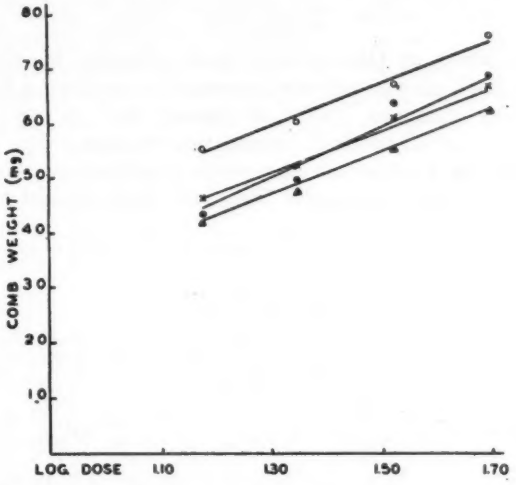

FIG. 1. Linear relationship between the comb weight in mg. and the logarithm of the dose of androsterone.

varied but their differences have been found significant only for doses above 50 μg.[2] The test of the linearity of the response for the doses below 15 μg. is shown in Table 2.

TABLE 2. TEST OF LINEARITY FOR THREE DOSES OF ANDROSTERONE

Term	Fact. coef. (x) for dose			Divisor $NS(x^2)$	Sum of products $S(xY_p)$	Variance $\frac{S^2(xY_p)}{NS(x^2)}$	F
	3.5	7.0	14.0				
Linear	−1	0	+1	22	140.5	897.28	13.42
Quadratic	+1	−2	+1	66	−7.5	0.85	0.01
Total Y_p of 11 responses on each dose	312.5	386.5	453	Error: $s^2 = 66.85$		$s = 8.17$	

The usual method of referring the activity of the unknown in terms of a standard preparation is to find the value of M called the logarithm of the ratio of potency. In the present assay and through the factorial analysis shown in Table 3, $M = \bar{1}.9188$ and $s_m = .0147$. Following the equations and the steps given by Bliss and Marks (1939) it was found that the renal excretion of androgenic material

[2] A detailed statistical analysis of the data here reported will appear later.

by the male cebus monkey was equivalent to 194 ± 14 μg. of androsterone per liter of urine. As the daily output of urine averages 130 cc. it follows that in these South American monkeys the renal excretion of androgens per 24 hours was equivalent to 25 μg. of androsterone.

COMMENTS

As the position of the straight lines relating the effect to the logarithm of the dose varies from one assay to another, a fact already pointed out by Emmens (1939), it follows that in each assay the concomitant use of a standard preparation is obligatory. It was also observed that the variance of the comb weights increased with the dosage. This is a disadvantage of the chick comb weight method but

TABLE 3. FACTORIAL ANALYSIS OF THE ASSAY OF ANDROGENS IN CEBUS URINE

Treatment effect	Fact. coef. (x) for dose				Divisor $NS(x^2)$	Sum of Prod. $S(xYp)$	Variance	Standard deviation	F
	S_1	S_2	U_1	U_2					
(1) Samples	−1	−1	+1	+1	44	−34.0	(D^2) 26.27	5.12	0.31
(2) Slope	−1	+1	−1	+1	44	132.0	(B^2) 396.00	18.96	4.68*
(3) Parallelism	+1	−1	−1	+1	44	1.0	0.02	0.14	
Total Y_p of 11 responses on each dose	386.5	453.0	370.0	435.5			s^2=84.46	s=9.19	

* Significant.

its accuracy can be maintained if the dosage levels for the unknown and for the standard are approximately the same and provided the total dose does not exceed 50 μg. Then, preliminary tests are necessary to ascertain the dose of the extract giving a reaction below that induced by 50 μg. of androsterone. With these qualifications in mind, the baby chick method seems useful and accurate for assaying the androgenic activity of an extract in terms of androsterone. The statistical principles here involved have been pointed out and applied also by Schild (1942) and Pugsley (1946).

The importance of studies on metabolism of the steroid hormones in primates is quite obvious. Anthropoid apes and rhesus monkeys have been frequently used but scarce data are available concerning the New World platyrrhine monkeys. Fish et al. (1941) reported an average of 680 μg/24 hrs. of androgenic material in the urine of the male chimpanzee. According to Dorfman and van Wagenen (1938) the rhesus excretes from 100 to 470 μg. during the same period. Although their quantitative assays are not exempt from criticism one sees that the androgenic material in cebus urine is very low. Preliminary studies have also shown no detectable amounts of 17-ketosteroids in extracts of urine from 10 days' collection.

It seems that the androgenic activity of urinary extracts reported until the present time, including those from men, must be checked according to the new lines of statistical principles. An attempt was made to follow them in this study.

SUMMARY

A linear relationship exists between the weight of the comb of the baby chick and the logarithm of the dose of androsterone applied locally in oil solution. Groups of 11–20 birds received from 3.5 to 168 μg. of the hormone and it was found that the slopes of the straight lines differ significantly only for doses above 50 μg.

Following Bliss and Marks' recommendations the chick comb weight method seems useful for the biological assay of androgens especially when the amount and the weak activity of the extract are restrictions to the use of capons.

The test was employed to determine the androgenic activity of urinary extracts of cebus monkeys. The renal excretion of androgens by this primate was found equivalent to approximately 25 μg./24 hours of androsterone.

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THE EFFECTS OF HYPOPHYSECTOMY ON SERUM AND STORAGE IRON IN ADULT FEMALE RATS[1]

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HYPOPHYSECTOMY produces a profound anemia in the dog (Aschner, 1912), in the rabbit (Houssay, Royer, and Orias, 1931), and in the rat (Stewart, Greep, and Meyer, 1935; Meyer, Stewart, Thewlis, and Rusch, 1937; Vollmer, Gordon, Levenstein, and Charipper, 1939; Crafts, 1941). Recently, it was shown that the anemia induced by hypophysectomy was of the microcytic hypochromic type in adult female rats (Crafts, 1946 a) and in the adult male rat (Crafts, 1946 b). Because of this microcytic hypochromic anemia and the finding that injections of iron, copper, and thyroxine would alleviate this anemia (Crafts, 1946 a), it was thought that hypophysectomy might influence the metabolism of iron. Accordingly, all phases of iron metabolism following hypophysectomy are being studied.

It is generally understood that a proper gastric acidity is necessary for iron absorption. Crafts and Walker (1947) recently reported that the hypophysectomized rat does not secrete as much gastric juice as a normal rat. The present report is concerned with the amount of iron in the serum and in storage in hypophysectomized adult female rats.

METHODS

Adult female rats of 3–4 months of age and of the Long-Evans strain were used for this study.

Hypophysectomy was performed under ether anesthesia by the parapharyngeal route. Blood was obtained by heart puncture under ether anesthesia. Ether anesthesia has no effect on the cellular blood picture of the rat (Crafts, 1944). Erythrocyte counts were made with U. S. certified pipettes and the improved Neubauer counting chamber. Hemoglobin determinations were made on an Evelyn colorimeter.

Viviperfusions, whereby blood is removed from an organ and replaced by an iron free modified Tyrode's solution, were performed on all rats before organs were removed for iron determinations. The method of Copp and Greenberg (1946) was used. The importance of removing blood, which contains much iron in the form of hemoblogin, from an organ before iron determinations are made cannot be too greatly emphasized.

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Each rat was anesthetized and blood removed from the heart for serum analysis, erythrocyte counts, and hemoglobin determinations. Serum iron was determined according to the procedure of Barkan and Walker (1940). The animal was then viviperfused and its organs removed. They were carefully weighed and iron determinations made.

Our method for iron determinations on tissues was fundamentally that of Schaefer (1940), with certain modifications adapting the final colorimetric reading to the same calibration of the photoelectric colorimeter used in the serum iron determinations. To a weighed piece of tissue (0.2 to 1.5 grams wet weight), in a 200×25 mm pyrex tube graduated at 50 cc, was added 2 cc of concentrated sulfuric acid and 2 cc of concentrated nitric acid. The tissue was dissolved by heating on a sand bath, adding more nitric acid if necessary to destroy fat. The sample was then made up to 50 cc volume.

Aliquots (2 cc or spleen for liver, 10 cc or heart, kidney or muscle) were measured into similar tubes graduated at 25 cc and heated first on the sand bath and then over a microburner until all liquid except the sulfuric acid was driven off as indicated by the appearance of dense fumes. Further addition of nitric acid was necessary if charring occurred. When digestion was apparently complete, as indicated by a clear solution, 5 drops of 30 per cent hydrogen peroxide was added. The addition of hydrogen peroxide was repeated. The solutions were allowed to cool and 2 cc. of 2 per cent sodium sulfite solution added.

At this point we deviated from the procedure of Schaefer by utilizing the red color of the ferrous salt of o-phenanthroline for the final estimation. Since this complex is most stable at pH 4.5, the next step was the adjustment of the mixture to pH 4.5 by the addition of concentrated ammonia water. The disappearance of the blue acid color of thymol blue was the most satisfactory indicator for this adjustment. One cc. of buffered 1 per cent hydrazine solution was then added, followed by one cc. of 0.1 per cent o-phenanthroline solution (these solutions prepared as in the method for serum iron). After at least one hour's standing, the volume was made up to 25 cc. with water, and the tubes read in the photoelectric colorimeter with a 490 millimicron filter. The colorimeter had been previously standardized with known solutions containing 0.05 and 0.2 mg. of iron carried through the above procedure in triplicate.

It should be constantly kept in mind that iron is present on all exposed surfaces and extreme care must be taken to prevent contamination of samples with extraneous iron. Glassware was cleaned with nitric acid, tap water (ten rinses) and distilled water (five rinses) and stored in a dustless cabinet. All water used in the analyses was redistilled from phosphoric acid.

Rats were maintained on Rockland Farms complete rate diet plus fresh lettuce once a week.

RESULTS

Adult female rats in this study were divided into three groups, i.e., 21 normal controls, 10 hypophysectomized, and 5 pair-fed controls. The last group of rats were fed the same amount of food per day that the hypophysectomized rats consumed. These served as controls in determining whether results obtained were due to hypophysectomy or due to decrease in food intake which invariably follows removal of that gland.

The erythrocyte count of the normal controls (table 1) averaged 8.25 million cells per cu. mm. and the hemoglobin was 14.5 gm. per 100 cc. Forty days after hypophysectomy, the erythrocyte count averaged 6.01 million cells per cu. mm. and the hemoglobin had decreased to 10.1 gm. per 100 cc. The animals which were pair-fed showed a slight decrease to 7.47 million cells per cu. mm. and 12.8 gm. of hemoglobin.

After an anemia had developed in the hypophysectomized rats (40 days) and the pair-fed rats had been subjected to a decreased food intake for 40 days, the animals were autopsied as already described and iron determinations made on the serum, liver, spleen, both kidneys, heart, and a portion of the thigh muscles.

Serum Iron

The iron content of the serum of the normal control rats was found to be 0.1430 mg. per 100 cc of serum. Hypophysectomy induced a decrease in serum iron to 0.0464 mg. per 100 cc. In 3 rats no iron whatsoever was detectable with the methods used. This decrease, however, may be due to the decreased food intake of the operated animals for the pair-fed rats showed a similar decrease to 0.0500 mg. of serum iron per 100 cc. There was no serum iron detectable in 2 of these pair-fed rats.

Storage Iron

Liver—The normal control rats showed 7.60 mg. of iron per 100 gm. of liver tissue (table 1). This figure is close to 6.1 mg. per 100 gm. of liver tissue reported by Austoni, Rabinovitch, and Greenberg (1940) who also used the viviperfusion technique in their work. The hypophysectomized animals had 8.22 mg. but the large standard deviation would make this difference insignificant. The pair-fed rats had an unexplainable figure of 37.20 mg. of iron per 100 gm. of tissue. The amount of iron per liver was calculated (table 2) and found to be 0.619 mg. for the normal controls and 0.285 mg. for the hypophysectomized rats, a 54% decrease. This decrease approximately equals the difference in weight of the livers of the normal control rats (6493.9 mg.), and the hypophysectomized rats (3642.2 mg.). This is a 44% decrease in weight. Although the concentration of liver iron in the 2 groups of rats was practically the same, the total amount of liver iron in the hypophysectomized animals was decreased due to decrease in size of the liver.

Livers of the pair-fed animals also decreased in weight (table 2) but the total amount of liver iron was 1.461 mg. as compared to 0.619 mg. for the normal controls. This is in agreement with the high concentration of liver iron in the pair-fed group of rats (table 1).

Spleen—The average amount of iron in 100 gm. of spleen tissue in the normal control rats was 93.2 mg. (table 1), which is higher than

obtained by Austoni, et al. (1940) who reported 46.1 mg. Both the hypophysectomized and the pair-fed groups had an elevated iron storage in the spleen; the hypophysectomized rats, 174.87 mg. and the pair-fed rats, 134.8 mg. (table 1). The large standard deviation in the hypophysectomized groups is mainly due to very large amounts of iron in 3 of the 10 spleens, i.e., 243.0 mg., 304.0 mg., and 359.0 mg. In terms of concentration, therefore, both the hypophysectomized and the pair-fed animals had a greater amount of iron in the spleen than the normal control animals.

TABLE 1. THE ERYTHROCYTE COUNT, HEMOGLOBIN LEVEL, SERUM IRON, AND STORAGE IRON IN NORMAL, HYPOPHYSECTOMIZED, AND PAIR-FED ADULT FEMALE RATS
± = Standard deviation

	Normal rats 21	Hypophysectomized rats 10	Pair-fed rats 5
Erythrocytes—millions per cu. mm.	8.25 ± 0.51	6.01 ± 1.16	7.47 ± 0.83
Hemoglobin—gm. per 100 cc.	14.5 ± 0.91	10.1 ± 1.62	12.8 ± 1.00
Serum—mg. per 100 cc.	0.1430 ± .0680	0.0464 ± .0575	0.0500 ± .0476
Liver—mg. per 100 gm.	7.60 ± 9.25	8.22 ± 8.21	37.20 ± 10.9
Spleen—mg. per 100 gm.	93.2 ± 41.4	174.87 ± 111.0	134.8 ± 42.6
Heart—mg. per 100 gm.	4.95 ± 2.06	8.11 ± 1.78	8.14 ± 1.91
Kidneys—mg. per 100 gm.	5.53 ± 1.81	10.49 ± 1.42	10.07 ± 2.27
Muscle—mg. per 100 gm.	1.70 ± 0.59	1.78 ± 0.86	1.55 ± 2.26

Table 2 shows the weight and the total amount of iron per organ. In spite of the decrease in weight of the spleen from a normal control of 452.7 mg., to the hypophysectomized group averages of 241.2 mg., a 47% decrease, the amount of iron in the whole spleen of the 2 groups was practically the same, i.e., 0.331 mg. and 0.389 mg. These figures agree with the greater concentration of iron in the spleens of the hypophysectomized rats (table 1). Similar results were found in the pair-fed group of rats (table 2). Although there was a 52% decrease in weight of the spleens between the normal control group and the pair-fed group, there was only a 12% difference in the amount of iron. This also agrees with the figures in table 1. Although these data show very large standard deviations, the results are exactly the same as found in the heart and kidneys.

Heart—Hearts of the normal control rats had an average of 5.53 mg. of iron per 100 gm. of tissue (table 1). Austoni, et al. (1940) found 7.2 mg. per 100 gm. The hypophysectomized rats averaged 8.11 mg. and the pair-fed group, 8.14 mg., a 32% increase in iron concentration in both cases.

Table 2 shows that the normal control hearts averaged 703.8 mg. in weight while in the hypophysectomized group the hearts averaged 392.8 mg., a 44% decrease in weight. The amount of iron, however, in the 2 groups is practically the same, i.e., 0.035 mg. and 0.032 mg. respectively. Although the total amount of iron in hearts of the pair-fed group was about the same as the normal controls, there was a 32% difference in heart weight (table 2).

These figures agree with the greater concentration of iron found in the hearts of the hypophysectomized and the pair-fed animals than in the hearts of the normal control rats (table 1).

TABLE 2. THE WEIGHT AND TOTAL IRON CONTENT OF THE LIVER, SPLEEN, HEART, AND BOTH KIDNEYS OF NORMAL, HYPOPHYSECTOMIZED, AND PAIR-FED ADULT FEMALE RATS
± = Standard deviation

	Normal rats 21		Hypophysectomized rats 10		Pair-fed rats 5	
	Wt. in mg.	Iron in mg.	Wt. in mg.	Iron in mg.	Wt. in mg.	Iron in mg.
Liver	6493.9 ±723.0	0.619 ± .636	3642.2 ±577.0	0.285 ± .252	3915.5 ±400.0	1.461 ± .522
Spleen	452.7 ±157.0	0.331 ± .310	241.2 ± 55.0	0.389 ± .209	216.4 ± 37.0	0.292 ± .112
Heart	703.8 ±112.0	0.035 ± .018	392.8 ± 49.0	0.032 ± .028	479.5 ± 77.0	0.038 ± .010
Kidneys	1517.3 ±300.0	0.082 ± .029	746.9 ±470.0	0.102 ± .037	1018.7 ± 65.0	0.103 ± .020

Kidney—The kidneys of the normal control group contained 5.53 mg. of iron per 100 gm. of tissue. (Austoni, et al., found 4.3 mg. per 100 gm.). As found in the spleen and heart, the hypophysectomized animals and the pair-fed rats had a higher iron concentration, i.e. 10.49 mg. and 10.07 mg. respectively (table 1).

The data shown in table 2 are in agreement with these findings. The kidneys weighed 1517.3 mg. in the normal controls but only 746.9 mg. in the hypophysectonized rats, a 51% decrease in weight. The iron content was not decreased, in fact, slightly increased from 0.082 mg. to 0.102 mg. The pair-fed rats, when compared with normal rats, showed a 33% decrease in weight of the kidneys but an increase in total iron from 0.082 mg. to 0.103 mg.

Muscle—Table 1 shows that there were no significant differences in the concentration of iron in the muscles between the control and the hypophysectomized rats or the pair-fed rats. All of these values would appear to be normal and are in close agreement with the figures reported by Austoni, et al. (1940) of 1.8 mg. per 100 gm. of muscle tissue.

In summary, these data show that the hypophysectomized rat has a decreased serum iron; that storage iron, in terms of concentra-

tion, is definitely elevated; that storage iron, in terms of total available iron, is normal except for the liver content. These data also show that these effects can be duplicated by decreasing the food of normal rats to the level consumed by the hypophysectomized rats. In spite of this similarity between the hypophysectomized rats and their pair-fed controls, the hypophysectomized rats exhibit a greater lack of ability to utilize the available stored iron in the formation of hemoglobin.

DISCUSSION

The hypophysectomized rat has a decreased serum iron but the iron concentration in the various organs is normal or increased. Whether these effects are due to the decreased food intake or not, the hypophysectomized rat has a decreased ability to use the stored iron in the formation of hemoglobin.

This is further substantiated by the finding that hypophysectomized rats treated with thyroxine alone or in combination with iron and copper maintained a normal or hyperplastic bone marrow and a normal or elevated erythrocyte count but did not exhibit a normal hemoglobin level (Crafts, 1946 a).

As there seems to be a normal supply of iron available, is decreased ability of the hypophysectomized rat to form hemoglobin due to a lack of protein to combine with the available iron pigment complex? Hahn and Whipple (1939) have shown that a decreased protein intake does limit hemoglobin formation in the dog in the presence of an adequate supply of iron. There is certainly a decreased protein intake in the hypophysectomized rat due to refusal to consume a normal amount of food. That hypophysectomy induces a disturbance in protein metabolism is well known. The decrease in total protein found in the hypophysectomized dog (Goldberg, 1938a & b) and in the hypophysectomized rat (Levin and Leathem, 1942; Levin, 1943) was reported as due to a decrease in the albumin fraction. There is, therefore, a decrease in available protein for formation of hemoglobin in the hypophysectomized rat. Pair-fed animals, given the same amount of food as consumed by hypophysectomized rats, would seem to be able to produce hemoglobin better than the hypophysectomized animals, as indicated by the difference in hemoglobin levels. As Levin (1943) pointed out, the normal pair-fed rat was able to utilize available endogenous protein while the hypophysectomized animals were not able to do so. Decrease in available protein or an inability to utilize protein may explain the lack of hemoglobin formation in the hypophysectomized animal.

Although no sign of infection occurred in these rats, the distribution of iron following hypophysectomy resembles that following infections in the human (Cartwright, Lauritsen, Jones, Merrill, and Wintrobe, 1946) and in the dog (Cartwright, Lauritsen, Humphreys, Jones, Merrill, and Wintrobe, 1946). These authors introduce a theory

that, although there is available iron in storage during infections, this iron is not utilized to combine with protoporphyrin to form heme because the iron is diverted to the tissues where it is made unavailable for hemoglobin formation. That a similar occurrence would follow hypophysectomy is a possibility.

SUMMARY

Adult female rats of the Long-Evans strain were divided into 3 groups, i.e., normal controls, hypophysectomized, and pair-fed controls given the same amount of food as consumed by hypophysectomized rats. Iron determinations were made on serum, liver, spleen, heart, both kidneys, and muscle. All organs were viviperfused before determinations were made. Serum iron was decreased after hypophysectomy but storage iron was increased. Although iron was available, the hypophysectomized rats did not produce a normal amount of hemoglobin. The pair-fed animals exhibited similar results although these animals were able to produce hemoglobin to a greater degree than hypophysectomized rats.

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THE SIGNIFICANCE OF THE ENDOCRINE GLANDS IN THE RIPENING OF RETICULOCYTES

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IT HAS BEEN shown in previous investigations that one or more substances capable of acting upon (accelerating) the development of reticulocytes are present in the blood (Plum, 1942).

Reticulocytes are, as is known, the only form of unripe red blood corpuscle which is normally present in the circulating blood. If the blood is drained from the vascular system and its coagulation prevented, a decrease in the number of reticulocytes can be observed (Pepper, 1922; Seyfarth, 1927; Heath and Daland, 1930; Heilmeyer and Westhauser, 1932).

On the basis of these earlier investigations, one of us (Plum, 1942) investigated as to whether the ripening of reticulocytes into erythrocytes takes place naturally through factors of their own, or whether they are influenced by substances in the surrounding liquid. It was shown that whereas reticulocytes in vitro in an 0.9% solution of sodium chloride at 40° decreased very slowly, the speed of this decrease was noticeably greater when liver extract was added to the solution or when the reticulocytes were mixed in plasma. The youngest and most unripe forms disappeared first, which can be taken to imply that it is an actual ripening process because the reticulocytes ripen into "adult" erythrocytes. This process of ripening follows the equation for the rate of a monomolecular reaction, the constant in this equation being proportionate to the concentration of ripening substances in the liquid surrounding the reticulocytes and increasing with rising temperature.

For the investigations reticulocytes were obtained from rabbits brought into a state of chronic anemia by daily blood lettings which was accompanied by reticulocytosis of from 20 to 30%. The monomolecular constant will always be the same when blood from the same rabbit is used and when the same concentration of ripening substances is used, but will vary from rabbit to rabbit. It was therefore necessary to introduce a standard in order to obtain comparable results. As such was used a 1% solution of liver extract of a certain batch, and the ripening constant of this in proportion to ox plasma has been established from the average of 11 determinations. The content of ripening substances in ox plasma was chosen as a unit, because ox plasma has the highest content of ripening substances found in normal plasma.

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For further details of the technique used in determining this constant refer to Plum, 1942.

In 1942 Jacobsen and Plum succeeded in dividing liver extract into a thermolabile and a thermostabile fraction, the latter, being identical with tyrosin, has in itself only little ripening effect but is capable of activating the thermolabile fraction. The activating power of tyrosin upon the reticulocyte ripening substances has since been investigated (Jacobsen and Plum 1942), and it was shown that in rabbits where the reticulo-endothelial system was blocked a decreased amount of ripening substances in plasma was observed, but by adding tyrosin the effect of plasma upon reticulocytes could be as great as the effect of plasma from normal animals, whereas adding tyrosin to normal plasma had no effect. It was later shown (Plum 1944) that the effect of organ extracts—liver, spleen, bone marrow, stomach—was accentuated by adding tyrosin.

Investigations on the ripening index of reticulocytes in normal human beings (Plum 1942) showed that except in women during the menstruation period, a low percentage of reticulocytes is proportionate to a high reticulocyte index and vice versa, this also being the case in certain categories of animals. Menstruation influences considerably the height of the ripening index and just before menstruation an increase in the quantity of reticulocyte ripening substances is seen.

As a close relation between the number of reticulocytes and the quantity of ripening substance in normal human beings was seen, a series of investigations into the quantity of ripening substances under pathological conditions was carried out.

In 1943 Jorgensen and Plum observed in a patient with pernicious anemia a decreased quantity of ripening substance in the plasma, which could however be activated by adding tyrosin. After adequate treatment with liver (Hepsol fortior "Mco") normal quantities of ripening substances were found.

On the strength of these observations Plum (1943) carried out an extensive series of investigations and showed that an alteration in the quantity of ripening substances was seen in certain pathological and physiological conditions; a decrease in the case of pernicious anemia, goitre and gastric ulcer; and an increase in myxedema, jaundice and pregnancy.

On account of the above mentioned clinical observations it was found expedient to investigate experimentally the part played by the endocrine system in the content of ripening substances in the blood, stomach and liver. At the same time a hematological status was carried out as will be seen from the following:

METHOD

For this investigation adult albino rats were used, and the weight of the rats at the beginning of the experiment is given in the attached tables. Un-

less otherwise stated, only male rats were used. The animals were kept in individual cages and were given the diet normally used in this laboratory consisting of:

Powdered Skimmed Milk	32.2 %
Potato Flour	43.1 %
Dried Yeast	8.1 %
Peanut Oil	16.2 %
Shark Liver Oil	0.27%

Hypophysectomy was carried out by the para-pharyngeal method as described by Smith. (1926).

The completion of the hypophysectomy was secured by examining sella turcica and its surroundings under lenses. The curve of body weight, the weight of the testis and suprarenal glands were a criterion as to the completeness of the operation, the experiments not being carried out until one month after the operation.

Castration of the male rats was carried out by the trans-scrotal method.

Ovariotomy and epinephrectomy were performed through lumbar incision (Schultzer, 1935).

After the operation the epinephrectomized rats were given 1% salt water ad libitum.

The thyroid glands were removed under lenses taking care not to injure the recurrent laryngeal nerves.

By means of Krogh's spirometer the basal metabolic rate was determined. As to the details of this apparatus refer to Krogh and Lindberg (1931).

The metabolism was measured after 24 hours of fasting.

The hematological examinations were carried out on the blood from the tail Plum (1944) and on blood from the caval vein in the case of newly killed animals.

The hemoglobin percentage was determined on a Zeiss Ikon hemometer, erythrocyte and leucocyte determinations were carried out in the usual way (Gram, Iversen and Meulengracht (1937). The reticulocyte count was made by the wet method (Plum 1944).

Experiments not yet published have shown that the reticulocyte counting can be done much more exactly than would be expected if reticulocytes and erythrocytes were mixed at random. This can be confirmed even by photographs. An experienced counter of reticulocytes should be able to reproduce reticulocyte numbers of 30% with an accuracy of $\pm .5$ to 1 per cent. This can be tested by giving the counter two series of countings with respectively 30% and 32%. He should be able clearly to distinguish between every counting in both series. It is of no use to make ripening experiments before the technical assistant is able to count with the exactness mentioned here.

In regard to the determination of the content of reticulocyte ripening substances of the blood, liver and stomach, refer to previous articles where the technique used is described in detail (Plum 1942).

RESULTS

The results of the experiments on hypophysectomized rats are given in Table I. As is seen from the table all animals were examined one month after the operation.

TABLE I. RESULTS OF HEMATOLOGICAL RESEARCHES AND RESEARCHES REGARDING RETICULOCYTE-RIPENING SUBSTANCES IN BLOOD, LIVER AND STOMACH FROM HYPOPHYSECTOMIZED RATS

Hypophysectomized Date	Killed	Weight in g	Hgbl. %	Erythrocytes in millions	Leucocytes	Reticulocytes %	Blood		Liver		Stomach	
							Ripening index	Ripening index activated	Ripening index	Ripening index activated	Ripening index	Ripening index activated
11/9	12/10	216 275	108 117	— 10.950	— 8720	— 22	— 0.83	—	—	—	—	—
11/9	12/10	191 155	109 103	8.030 8.540	2800 14840	6 12	— 0.64					
16/9	12/10	204 154	112 92	8.770 7.670	8000 16000	5 10	— 0.68					
20/9	21/10	200 134	107 86	7.270 5.950	11200 14640	8 15	— 0.66	0.81	0.21	0.88	0.11	0.98
23/9	21/10	209 151	109 96	11.660 9.400	14880 16680	14 31	0.64	0.81	0.18	0.90	0.10	1.00
23/9	21/10	308 155	109 102	8.230 5.820	12120 15840	18 35	0.63	0.83	0.20	0.90	0.11	1.00
24/9	23/10	200 155	— 98	— 6.290	— 14360	— 12	0.61	0.82	0.23	0.90	0.12	1.00
24/9	23/10	200 142	— 79	— 8.650	— 12840	— 17	0.60	0.83	0.22	0.92	0.10	1.00
27/9	25/10	199 157	111 84	— 8.540	15780 16480	15 25	0.68	0.82	0.19	0.87	0.09	0.98
29/9	25/10	206 139	116 78	10.390 9.510	13400 9160	22 32	0.66	0.84	0.20	0.92	0.09	1.00
29/9	4/11	199 143	111 56	9.140 3.120	16640 9320	23 32	0.65	0.82	0.19	0.92	0.10	1.04
1/10	4/11	204 146	103 87	9.160 8.890	10200 10640	15 28	0.62	0.82	0.17	0.92	0.09	0.96
1/10	4/11	203 147	100 72	11.210 8.420	13200 12840	16 18	0.63	0.84	0.17	0.96	0.10	1.02

4/11	147	72	8.420	12840	18	0.63	0.84	0.17	0.96	0.10	1.02

TABLE II. RESULTS OF HEMATOLOGICAL RESEARCHES AND RESEARCHES REGARDING RETICULOCYTE-RIPENING SUBSTANCES IN BLOOD, LIVER AND STOMACH FROM 6 NORMAL RATS

Date	Weight in g	Hgbl. %	Erythro-cytes in mill.	Leucocytes	Reticu-locytes %	Blood		Liver		Stomach	
						Ripening index	Ripening index activated	Ripening index	Ripening index activated	Ripening index	Ripening index activated
12/10	257	96	883	11200	6	0.86	0.88	0.27	0.89	0.18	1.07
23/10	209	88	789	9460	8	0.82	0.85	0.25	0.88	0.16	1.04
25/10	237	102	923	14880	5	0.82	0.84	0.26	0.90	0.20	1.07
4/11	267	98	902	9840	10	0.77	0.80	0.25	0.90	0.17	1.05
4/11	243	87	856	12860	7	0.82	0.86	0.29	0.92	0.19	1.06
6/11	206	109	900	8860	9	0.79	0.84	0.27	0.90	0.17	1.02

By comparison with the controls (Table II) it is seen that all the hypophysectomized rats showed a considerable decrease in the quantity of ripening substances in the plasma, but normal functioning of the plasma could be affected by adding tyrosin.

Only in rat No. 1 were normal conditions found, but this rat was in fact incompletely hypophysectomized and post mortem showed that more than half of the pituitary gland remained.

TABLE III. HEMATOLOGICAL ALTERATIONS IN CASTRATED RATS

No.	Operation	Hgbl. %	Erythrocytes in mill.	Leucocytes	Reticulocytes %	Ripening index	Ripening index activated	Date of investigation
Male								
1642	28/10	96 89	857 702	9720 16840	8 10	— 0.78	— 0.84	26/10 22/11
1643	28/10	89 90	810 861	12720 17440	7 10	— 0.84	— 0.87	26/10 22/11
1644	28/10	94 83	— 958	12800 13880	8 8	— 0.81	— 0.84	26/10 23/11
1645	28/10	95 90	847 950	11400 10400	6 8	— 0.84	— 0.85	26/11 23/11
1646	28/10	100 90	875 819	11860 13840	11 18	— 0.83	— 0.87	26/11 23/11
Female								
332	27/10	118 91	889 883	8200 16840	10 12	— 0.81	— 0.84	27/10 24/11
333	27/10	90 89	 855	 14560	20 20	— 0.82	— 0.84	27/10 24/11
334	27/10	118 94	1191 677	10920 8960	4 6	— 0.81	— 0.85	28/10 25/11
335	27/10	111 96	798 725	13200 13360	5 8	— 0.80	— 0.84	28/10 25/10
336	27/10	110 98	712 695	15880 13480	6 5	— 0.82	— 0.85	28/10 25/11

In the liver and stomach of the hypophysectomized rats a decrease in the quantity of ripening substances was observed, but as in the case of plasma, the organ extracts regained quite normal activity when tyrosin was added (Table I).

In order to see if these changes in the content of ripening substances in the blood, stomach and liver were due to changes of other endocrine glands as a result of hypophysectomy, or to the extirpation of the pituitary gland itself, castrated, epinephrectomized and thyreodectomized rats were examined.

As shown in Tables III and IV, no changes were found among the animals which were castrated and epinephrectomized. After thyreodectomy however conditions are seen to be identical with the conditions after hypophysectomy (Table V).

One of us has in another work (Christensen 1945) determined the standard metabolism of 70 normal adult male rats. It was shown that the rate was between 100 and 120 cc. oxygen per minute, per square

meter surface. Only in 4 rats were different results found (95–98–123 and 125 cc. oxygen).

After hypophysectomy the same rates of metabolism were observed as after thyreodectomy. In these animals the rate of metabolism was 60–75 cc. oxygen (23 rats in all).

Of the rats used in this investigation, three (Nos. 2, 7 and 8), had a remarkably high rate of metabolism (between 80 and 90 cc. oxygen). In only one of these rats did we succeed in finding glandular tissue in the surroundings of the thyroid gland by microscopical examination,

TABLE IV. HEMATOLOGICAL ALTERATIONS IN EPINEPHRECTOMIZED MALE RATS

No.	Operation date	Hemoglobin %	Erythrocytes in mill.	Leucocytes	Reticulocytes %	Ripening index	Ripening index activated	Date of investigation
1647	28/10	100 94	820 855	13400 20600	9 11	— 0.81	— 0.83	26/10 20/11
1648	28/10	104 94	834 827	10320 17000	12 10	— 0.82	— 0.83	26/10 20/11
1649	28/10	109 94	816 782	11680 15720	10 15	— 0.80	— 0.83	26/10 20/11
1650	30/10	102 86	914 934	12400 19360	6 6	— 0.78	— 0.82	26/10 22/11
1651	30/10	109	820	11200	5	Died spontaneously after few days		

but it is highly possible that all three rats have only been partially thyreodectomized and accordingly we found in these rats a somewhat higher ripening index than among the other rats.

DISCUSSION

In agreement with Vollmer et al. (1939) and Meyer et al. (1940) anemia combined with a decreased number of erythrocytes was found in most of the rats after hypophysectomy but the changes were however not so pronounced as those found by the mentioned investigators.

Further it was found that the number of leucocytes was not changed in a characteristic way after hypophysectomy.

As to the reticulocytes our findings were completely different from those of the other investigators.

While Vollmer et al. (1939) and Stewart et al. (1935) find the number of reticulocytes in normal rats at between 6–48% and Overbeck (1936) finds 0–16% and all of them finding a considerable decrease in the reticulocyte number after hypophysectomy, we find that the number of reticulocytes in normal rats is between .4 and 2%, increasing after hypophysectomy (1–4%) and after thyreodectomy (4–7%).

Investigations of the content of ripening substances in the blood and in the organs show that the changes are the same after hypophysectomy as after thyreodectomy.

TABLE V. RESULTS OF HEMATOLOGICAL RESEARCHES AND RESEARCHES REGARDING RETICULOCYTE-RIPENING SUBSTANCES IN BLOOD, LIVER AND STOMACH AFTER THYROIDECTOMY

Thyroidectomized	Metabolism ml. O_2 per min. per m^2 surface	Killed	Weight in g	Hemoglobin %	Erythrocytes in mill.	Leucocytes	Reticulocytes %	Blood		Liver		Stomach	
								Ripening index	Ripening index activated	Ripening index	Ripening index activated	Ripening index	Ripening index activated
4/12	(17/12):73		174	—	—	—	—						
		20/12	143	97	11.620	15.160	60	0.66		0.17	0.92	0.11	0.99
4/12	(17/12):90		176	—	—	—	—						
		20/12	193	100	10.916	19.080	28	0.72		0.20	0.91	0.11	1.02
4/12	(29/12):70		171	—	—	—	—						
		11/1	168	100	8.770	14.400	45	0.67	0.81	0.19	0.88	0.10	1.03
7/12	(29/12):66		137	—	—	—	—						
		11/1	146	92	9.280	13.840	44	0.62		0.17	0.96	0.08	0.96
7/12	(29/12):75		143	—		—	—						
		11/1	156	80	7.850	14.040	59	0.64	0.84	0.20	0.94	0.09	0.99
7/12	(30/12):73		172	—	—	—	—						
		14/1	163	90	8.090	15.000	73	0.61		0.19	0.89	0.09	1.02
9/12	(30/12):79		161	—	—	—	—						
		14/1	194	99	7.100	17.040	53	0.68		0.21	0.91	0.10	0.98
9/12	(30/12):83		160	—	—	—	—						
		17/1	198	80	8.310	17.480	47	0.74	0.81	0.22	0.90	0.11	1.00

There is therefore reason to suppose that the observed decreased ripening index after hypophysectomy is due to a decreased or eliminated function of the thyroid gland, even if one cannot exclude the possibility that the changes after thyreodectomy are due to damaging the parathyroid gland, which is always removed or damaged by thyreodectomy.

Assuming that it can be taken for granted that the parathyroid glands are nor regulated by the pituitary gland, this possibility can be disregarded, and only the fact remains that after hypophysectomy as well as after thyreodectomy an alteration in content of reticulocyte ripening substances in the blood is found. This alteration takes place at the expense of the already formed ripening substances and at the same time the quantity of potential (which tyrosin can activate) substances increases. Thus it seems clear that the thyroid gland produces substances which are of importance to the activation of the ripening substances in the organism, and that this function is controlled by the hypophysis.

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EFFECTS OF LOW BAROMETRIC PRESSURE ON THE CHEMICAL COMPOSITION OF THE ADRENAL GLANDS AND BLOOD OF RATS[1]

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WHILE it is generally agreed that the adrenal cortices play a prominent role in the adaptation of animals to anoxia, the mechanisms involved in the activation of these glands in this circumstance have not yet been described. It is possible that a fuller understanding of these mechanisms will only be achieved when future investigation reveals a more complete picture of the metabolic function of the adrenals than is available at present. However, the technique of testing the biochemical responses of animals to graded "stress" stimuli may prove itself to be a useful tool in elucidating the mechanism of activation of the adrenals, and subsequently, the function of their hormonal products in metabolism. Exposure of animals to varying degrees of anoxia has proved to be a convenient method of administering quantitatively graded stimuli.

Adrenal cortical hypertrophy as a result of anoxia was described by Armstrong and Heim (1938) and others (see Hailman, 1944, for review). That this hypertrophy is probably accompanied by an increased output of hormone is suggested by the work of Langley and Clarke (1942), who found that adrenalectomized animals maintained at a simulated altitude of 20,000 feet require many times the sea level maintenance dose of cortical extract for survival.

Histochemical studies of the adrenal cortex in many "stress" circumstances, including that of anoxia (Darrow and Sarason, 1944), have revealed that the first detectable change in the adrenals of animals subjected to stress is a depletion of lipoid, usually in the perimedullary portion of the gland. These changes are frequently visible within a few hours after subjecting an animal to anoxia, whereas it is often difficult to detect hypertrophy of the gland at the end of 24

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hours of exposure. Man and Tepperman (1942) found that the cholesterol content of the adrenals of rats exposed to a simulated altitude of 20,000 feet for 24 hours was reduced by 70%, but that the fatty acid content of the glands was unchanged. Knouff, Brown and Schneider (1941) had found a similar reduction in adrenal cholesterol in guinea pigs which had exercised violently.

The following experiments were done to test the feasibility of using depletion of adrenal gland cholesterol as an index of adrenal activation in acute experiments. In addition to the total and free cholesterol and ascorbic acid content of the adrenals, pertinent chemical studies were made on plasma and whole blood.

MATERIALS AND METHODS

Sprague Dawley male rats weighing approximately 200 grams were used in all experiments. They were fed a diet of Purina Chow *ad libitum* and were fasted for 24 hours before decompression. Groups of four rats were kept at reduced pressures in a large, well-ventilated decompression chamber for five hours at 75–80° F. At the end of that period the rats were anesthetized with pentobarbital and blood samples were obtained from the heart in the decompression chamber at altitude. Tungstic acid filtrates of heparinized whole blood for lactic acid and glucose estimations were prepared immediately. The animals were then recompressed and the adrenals were removed and weighed. One adrenal from each animal was analyzed for cholesterol and the other for ascorbic acid.

Lactic acid was estimated colorimetrically by the method of Barker and Summerson (1941). Glucose analyses were carried out according to the method of Somogyi (Peters and Van Slyke, 1932). The method of Frame, Russell and Wilhelmi (1943) was used to estimate the plasma amino acid nitrogen in tungstic acid filtrates. Free and total cholesterol analyses of plasma and of adrenal gland were performed by the method of Schoenheimer and Sperry as described by Sperry (1942). The Bessey and King modification of Tillman's method was used to estimate adrenal ascorbic acid (Hawk and Bergeim, 1937).

EXPERIMENTAL RESULTS

Adrenal Cholesterol

The total and free cholesterol content of the adrenal glands is presented graphically in Figure 1. It is apparent that, up to 25,000 feet, there is a curvilinear fall in adrenal cholesterol with increasing altitude, and that this fall is accounted for entirely by changes in the esterified fraction. The total cholesterol at 25,000 feet (2.46 per cent) is about 45 per cent of the sea level control value of 5.48 per cent. The 28,000 foot mean (2.78 per cent) deviates strikingly from the curve, since the predicted value for that altitude from the previously ascertained curve would have been approximately 1.3 per cent. The deviation of this point is interesting in the light of the results of the blood and plasma studies to be reported below. It is especially noteworthy that there was no evidence of adrenal hypertrophy in any of the exposed group of animals.

Adrenal Ascorbic Acid

The ascorbic acid content of one adrenal of each animal was estimated, since it has been suggested that the ascorbic acid level may vary with the activity of the gland (Giroud et al., 1940; Mulinos et al., 1942). In addition, Sayers, Sayers, Lewis and Long (1944) have shown that the injection of purified pituitary adrenotropic hormone results

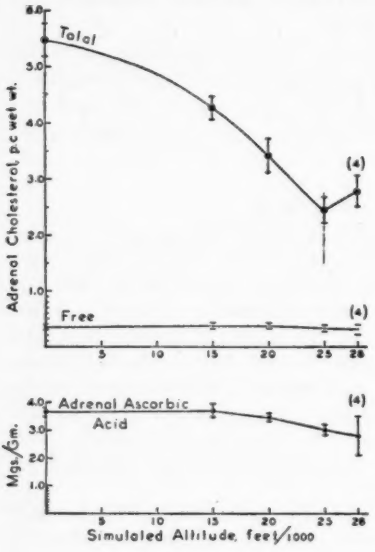

FIG. 1.

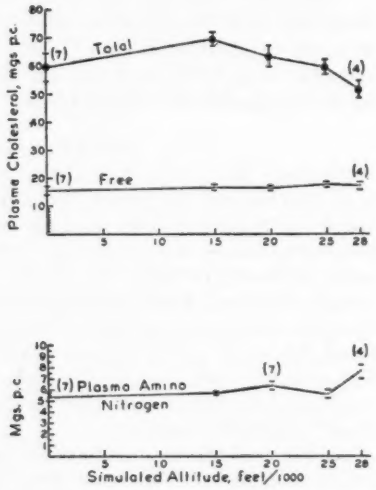

FIG. 2.

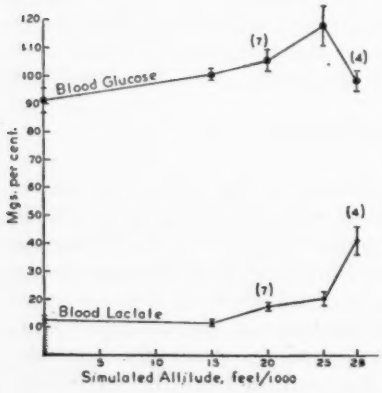

FIG. 3.

FIGS. 1, 2, 3.—Changes in adrenal gland and blood constituents observed at various simulated altitudes. All entries represent the mean and standard error of the mean for groups of eight rats. Exceptions are designated by a numeral in parentheses which signifies the number of rats per group.

in a lowering of adrenal cholesterol in the rat. The present study revealed that, although the changes in adrenal cholesterol were rather large, those in ascorbic acid were relatively small (see Figure 1). It is impossible to deduct from these data that the cholesterol depletion and the ascorbic acid depletion are primarily related phenomena, as they are believed to be by Long (Sayers, Sayers, Lewis and Long, 1944). The construction of a time curve of response to a single degree of anoxia might provide a useful datum in the consideration of this problem.

Plasma Cholesterol

The maintenance of a constant level of cholesterol ester in the plasma is, in part, dependent upon continuing esterification of cholesterol by the liver. Plasma cholestrol concentrations were estimated in these experiments because it was believed that the esterification of cholesterol probably requires the expenditure of oxidative energy. The altitude response curve in Figure 2 indicates that this may be so.

The mean plasma cholesterol at sea level shows a larger standard error than do the means at altitude, since the sea level group was weighted by three unaccountably low values. The mean of the 15,000 foot group (69.5 mgs. per cent), however, showed a very small standard error and agreed better with values obtained on similar animals in other laboratories. For this reason little significance is attached to the apparent trend toward a higher level at 15,000 feet than at sea level.

The esterified cholesterol of the plasma shows a tendency to drop progressively at 20,000, 25,000 and 28,000 feet, the change being most marked in the last 3,000 feet. The difference in the 15,000 foot level (69.5 mgs. per cent) and the 28,000 foot level (51.5 mgs. per cent) is regarded as statistically significant. The abrupt change at the critical altitude of 28,000 feet is interpreted as evidence of impairment of hepatic function by anoxia.

Plasma Amino Acid Nitrogen

In recent studies of stagnant anoxia, as encountered in hemorrhagic shock, attention has been drawn to the elevation of blood or plasma amino acid nitrogen in the shocked animals (Engel, Winton and Long, 1943). The maintenance of a normal concentration of amino acids in the plasma is dependent upon an adequately functioning liver. It is of some interest, then, that when the animals were strikingly anoxic at 25,000 feet and when a relatively large depletion of adrenal cholesterol had already occurred, their liver tissue was still competently deaminating amino acids at a sufficiently rapid rate to keep the plasma amino acid level within normal limits (Figure 2). It is only at the critical altitude of 28,000 feet that a clearcut trend toward elevation of the plasma amino acid level occurs.

Blood Glucose

Blood glucose concentrations were often rather variable, particularly in the 25,000 foot group of animals. Up to that altitude there was a gradual and progressive tendency toward hyperglycemia, but again the 28,000 foot level deviated abruptly from the curve established by the preceding points. Presumably, the hyperglycemia in this circumstance is related to adreno-medullary stimulation, although the animals had been fasted for 24 hours and their liver glycogen content was therefore relatively low at the onset of each experiment.

Blood Lactate

Estimation of the blood lactate affords a measure of the extent to which the animal is forced to depend on anerobic metabolic pathways for its energy supply. It was found that the blood lactate level was no higher at 15,000 feet than at sea level. At 25,000 feet the concentration of lactic acid in the blood was about 20 mgs. per cent. Between 25,000 and 28,000 feet, however, there was a sharp rise to about 40 mgs. per cent.

"Clinical" Condition

The four most priminent signs observed in the exposed animals were hyperventilation, cyanosis, lethargy, and incoordination of movement which, in the case of the 28,000 foot group, was practically a generalized paralysis. Although the animals at 15,000 feet may well have been hyperventilating to some extent, their general appearance and behavior did not differ greatly from that of the sea level controls. At higher altitudes, however, all of the symptoms were clearly apparent and became progressively more pronounced with increasing altitude.

A very striking sign encountered in all of the animals above 15,000 feet was marked hyperreflexia. This was discovered when the rats were being tested for the efficacy of the pentobarbital anesthetic. At 20,000 feet the response to tail- or paw-pinching was far greater than it was at sea level. At 25,000 feet and 28,000 feet some animals leaped six or eight inches above the table in response to pinching of the tail. Although an effort was made to keep the animals under the anesthetic for 15 minutes before blood was taken from the heart it is possible that some of the excess of lactate in the blood at high altitude was related to the muscular exercise incident to hyperreflexia.

DISCUSSION

All of the chemical studies exhibited an orderly series of curves up to a stimulated altitude of 25,000 feet. The interval between 25,000 and 28,000 feet, however, was characterized by abrupt deviations from the previously established curves by the adrenal cholesterol ester content, the plasma cholesterol and amino acid nitrogen concentra-

tions, and the blood lactate and glucose concentrations. This is of some interest in view of the fact that practically all rats of strain, sex, age, and dietary history similar to those used in these experiments are capable of making a successful adaptation to an altitude of 25,000 feet, whereas 28,000 feet is often given as a survival ceiling at which a certain number of the animals fail to adapt (Dalton et al., 1944).

The significance of the depletion of cholesterol from the cortex is not well understood. Since the hormonal products of the adrenal cortex are steroids, it is entirely possible that cholesterol is the hormone precursor and that the diminution of cholesterol in the gland represents an accelerated conversion of cholesterol into hormone. (There has been striking proof that cholesterol is a precursor of at least one other biologically active steroid, since Bloch and his colleagues (1943) have demonstrated the conversion of cholesterol to cholic acid in the dog by means of deuterium-labelled cholesterol.) This hypothesis gains strength from the fact that the necessity for an intact hypophysio-adrenal axis for the characteristic adrenal cortex response to "stress" has often been demonstrated. Recently Sayers, Sayers, Fry, White and Long (1944) have discovered that the administration of their highly purified adrenotropic hormone of the anterior hypophysis to rats results in a striking decrease in adrenal cholesterol ester within three hours. Since, in this case, as well as in that of anoxia, there are corollary evidences of increased adrenal cortical activity (increase in fasting liver glycogen, increase in urinary nitrogen), the conclusion that depletion of adrenal cholesterol is accompanied by an increased rate of production of adrenal cortical hormone is inescapable.

Thus, the diminution of adrenal cholesterol seen in these experiments may indicate the extent to which the hypophyseal-adrenal system is called upon to provide extra hormone to the animal. The fact that the cholesterol content of the gland at 28,000 feet does not conform to the previously determined curve for lower altitudes may signify that the chemical reactions which the animal must perform in order to adapt to a low-oxygen environment are themselves dependent, in some measure, upon a continuing supply of molecular oxygen. This purely "operative" oxygen supply (Peters, 1941)—in this case, the oxygen that is necessary to support the intermediary reactions involved in adapting to the "stress" of anoxia—appears to be adequate up to the altitude of 25,000 feet. The aberrant value at 28,000 feet, then, probably represents a failure of the critical "operative" oxygen supply, or, by empirical definition, a failure to adapt successfully. This inadequacy of "operative" oxygen is apparent not only in the adrenal cholesterol but also in the plasma cholesterol and amino nitrogen levels, and in the blood glucose and lactate concentrations. These data constitute testimony of failing liver function on the one hand and a shift from aerobic to anerobic metabolism in the periphery on the other.

The altitude range in which failure to adapt occurs is a very narrow one. It is understood that the biochemical definition of this range described herein applies to rats of the weight, sex, strain, dietary and general environmental history of those used in this study. The results of preliminary experiments suggest that the altitude at which failure of the operative oxygen supply occurs may be determined, to some extent, by the previous dietary and environmental experience of the animals.

The mechanism of stimulation of the hypophyseal-adrenocortical system in anoxia is not clearly evident, although recent work by Langley and others (1943) and by Hailman (1944) suggests a most promising approach to the problem. Using an histochemical technique, Langley, Nims, Harvey and Clarke found that the prevention of acapnia by the administration of carbon dioxide prevented the characteristic depletion of lipoids in the adrenal cortices of rats exposed to low barometric pressure for 24 hours. Similarly, Hailman discovered that the administration of carbon dioxide in the inspired air of rats discontinuously exposed to anoxia for 5 days prevented the characteristic adrenal hypertrophy from occurring. Both of these investigators have suggested that the stimulus for adrenal hypertrophy in this circumstance is a disturbance of acid base balance and is probably related to an excess of base incident to hyperventilation. It is certainly true that at a simulated altitude of 20,000 feet striking hyperventilation and marked hyperreflexia were observed in conjunction with an appreciable fall in adrenal cholesterol ester, while studies of other blood constituents revealed only minor deviations from the normal values. Future understanding of the mechanism whereby the prevention of acapnia alters the response of the adrenals to anoxia will yield basic information concerning the physiology of these glands.

SUMMARY

The cholesterol ester content of the adrenal glands of rats exposed to varying degrees of anoxia for five hours was found to diminish in a curvilinear fashion with increase in altitude up to 25,000 feet. The mean sea level control value was 5.48 per cent whereas the value at 25,000 feet was 2.46 per cent. At 28,000 feet, however, the determined point failed to conform to the previously ascertained curve.

Simultaneous studies of adrenal ascorbic acid content, plasma cholesterol, plasma amino acid nitrogen, blood lactate, and blood glucose concentrations revealed small but progressive deviations from the sea level values up to 25,000 feet. The interval between 25,000 and 28,000 feet was characterized by more striking changes in the concentrations of most of the substances studied. The possible theoretical significance of these abrupt changes is discussed.

The experimental results are summarized graphically in Figures 1, 2, and 3.

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HORMONAL FACTORS RESPONSIBLE FOR DEPOSITION OF CHOLESTEROL IN THE CORPUS LUTEUM OF THE RAT

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AN HISTOCHEMICAL study of the lipids in corpora lutea of the rat at various stages of the estrous cycle (Everett, 1945, b) disclosed with advancing diestrus an increasing abundance of substances which give the Schultz reaction and which are regarded as cholesterol and its esters. A transitory depletion of these substances was noted during proestrus, followed by their renewed intracellular deposition during the stage of full cornification. Evidence was presented which indicated that such corpora lutea are weakly stimulated during diestrus, undergo a burst of activity during proestrus and then begin to retrogress after the new ovulation has taken place. The deposition during the post-ovulatory period of large amounts of cholesterol in the next youngest set of corpora lutea was shown to represent regression *after a moderate degree of luteotrophic stimulation.*

It seemed probable, however, that factors other than luteotrophin (lactogen)[1] are concerned in bringing about cholesterol accumulation. In "fully activated" corpora lutea of this species, in either pregnancy or pseudopregnancy, preliminary study had shown very little visible fat detectable by Sudan staining or by the Schultz cholesterol test. Estrogen seemed to be a likely candidate as an additional factor, since in cyclic rats cholesterol accumulation is in a general way associated with high estrogen levels. It had been noted that, when ovulation time was accelerated by the administration of estrogen in middiestrus (Everett, 1946), the corpora lutea from the previous cycle developed extensive deposits of Schultz-positive lipids 24 hours earlier than if no treatment were given. Accordingly it was postulated that estrogen, or some agent evoked by estrogen, acting in the presence of luteotrophic stimulation would result in accumulation of cholesterol in corpus luteum cells.

Preliminary tests of this possibility were performed by administering single injections of estradiol benzoate to pregnant rats (Everett, 1945, a). Not only were cholesterol-rich corpora lutea observed 48 hours later, but also newly ruptured follicles and tubal ova. Experi-

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[1] The expression "luteotrophin" (Astwood, 1941) will be employed freely in this paper, recognizing that the lactogenic hormone (Evans, et al., 1941) may well be the only luteotrophin acting in cyclic rats and early pregnancy. The term is preferred here because it directs attention to the particular effect upon the corpus luteum.

mental analysis of this mechanism is presented herewith, showing: (1) that the cholesterol response to estrogen is mediated by the hypophysis; (2) that not estrogen, but probably hypophyseal LH is the agent which effects cholesterol deposition in the presence of luteotrophin; and (3) that an excess of luteotrophin prevents this action of LH on the corpus luteum.

MATERIALS AND METHODS

Sexually mature albino rats of the inbred Vanderbilt (Osborne-Mendel) strain were used for this study. Vaginal smears were prepared regularly each morning for varying lengths of time before each experiment, depending upon the requirements of each case. In experiments with pregnant rats, each female was mated with a known fertile male during proestrus. If sperm were found in the smear the following morning, that date was reckoned as zero time. In pseudopregnancies, induced by mating with a vasoligated male, zero time was reckoned as the day following proestrus.

Hypophysectomy was performed by the parapharyngeal approach, the gland being removed by suction. Usually the pars nervosa and the pars anterior (in 2 pieces) were readily identified in the vacuum line after the operation. In all cases, at autopsy, the cranial floor at the former site of the gland was examined minutely under dissecting lenses for any remaining tissue. If fragments of doubtful nature were found and if the nature of the experiment demanded proof of complete anterior lobectomy, the material was fixed, serially sectioned and stained for identification.

Estradiol benzoate[2] was administered by subcutaneous injection of a sesame oil solution containing 0.33 mg. per cc., unless otherwise specified. The standard dose was 50 μg. (0.15 cc.). When smaller amounts were given, the stock solution was diluted with oil to give a final volume equivalent to the standard. In a number of experiments small crystals of estradiol[2] (ca. 50 to 200 μg.) were introduced by means of a 16 gauge hypodermic needle, using the stylus to propel the crystal into position beneath the skin. In other instances estradiol was administered in the form of subcutaneously implanted beeswax pellets containing approximately 100 μg. of estradiol and prepared according to a method previously described (Everett and Sawyer, 1946).

Two samples of lactogenic hormone[3] were employed, designated lactogen S and lactogen A, respectively. The latter was found to be contaminated with LH. Immature male rats (22 days old) were injected intraperitoneally twice daily for 4½ days and were killed the morning after the final injection (Fevold, 1937). Weights of seminal vesicles with coagulating glands and of anterior prostates were increased 45 to 50% after total doses of 100 I.U. of lactogen A. Lactogen S, on the contrary, gave no similar effect in total doses of 300 I.U.

Purified sheep hypophyseal LH (sample P118), obtained through the

[2] Generously contributed by Dr. Erwin Schwenk of the Schering Corporation, Bloomfield, N. J.

[3] Lactogen S was a highly purified powder (sample 48 Hy ex 4) obtained from the Schering Corporation. Lactogen A was a commercial preparation (lot 7641) which was kindly supplied by Dr. J. M. Scott of Ayerst, McKenna and Harrison, Ltd., New York City. Both preparations were administered in solution, containing 100 I.U. per cc.

courtesy of Dr. R. K. Meyer (Wisconsin), was assayed in his laboratory for both FSH and LH activity. In total doses of 3.3 mg. (0.5 gm. equivalent of the whole dried gland) no detectable FSH activity was found. On the other hand, such doses augmented ovarian stimulation by a standard FSH preparation (av. ovarian wts. with LH and FSH: 60 mg., against 45 mg. produced by FSH alone).

Most of the experiments were designed so that they would terminate on the sixth day of pregnancy. In such cases surgical and injection procedures were largely confined to the fourth or fifth days. Variability of response resulting from variation in stage of pregnancy was thus reduced to a minimum. At the end of each experiment the animal was killed with illuminating gas, after which the ovaries and tubes were removed to physiological saline for close examination. The occurrence of recent ovulation was demonstrated by direct recognition of newly ruptured follicles and of tubal ova. In visualizing the latter the ampullae of the oviducts were excised and placed in saline under a cover glass for microscopic observation. Portions of the ovaries containing the corpora lutea of the desired set were separated, each piece usually containing 2 or 3 luteal bodies. The tissue was fixed in 10% neutral formalin, subsequently washed, infiltrated with gelatin and sectioned on the freezing microtome. Routinely the sections were cut with the microtome set at 10 μ. In certain cases sections were also cut at 30 μ and are referred to in the text as "thick sections."

Representative sections were stained with Sudan III and others from the same corpora lutea were studied for cholesterol content by the Schultz technique. The reader is referred to the article on lipids in cyclic corpora lutea (Everett, 1945, b) for details of these procedures.

In selected cases the digitonin reaction was employed in a modified form in combination with the Schultz test, for estimation of free cholesterol (Everett, loc. cit., p. 304; cf. the Schoenheimer and Sperry (1934) technique where the digitonin and Liebermann-Buchard reactions are combined for cholesterol determination). Our method was as follows: Sections from the same piece of tissue were arranged in four groups. Group I was placed in 0.2% digitonin in 50% alcohol for 2 hours, extracted 30 minutes in acetone, washed briefly in 50% alcohol, before and after extraction, and then returned to water. Group II was similarly treated in digitonin, washed, and returned to water without extraction. Group III received no treatment with digitonin, but was extracted for 30 minutes in acetone as above. Group IV was neither treated with digitonin nor extracted. All four groups were separately placed in 2.5% ferric alum at 37° C. for 2 days. At the end of that time they were tested with the Schultz reagent. Groups II and IV regularly gave equivalent color reactions. The difference in intensity of blue-green coloration between groups I and III presumably represented the insoluble cholesterol digitonide retained in the former. A faint tinge of positive color in thick slices (30 μ) was usually found after prolonged acetone extraction of formalin-fixed, cholesterol-rich material although initial fixation in absolute alcohol, ether-alcohol or acetone resulted in complete extraction of sudanophile or Schultz-positive lipid.

Specificity of the Schultz test. Additional information pertaining to this question has been recently obtained. A full exposition is to be presented elsewhere, emphasizing that the Schultz test is closely related to the Lifschütz

"oxycholesterol" test and demonstrating that it is decidedly more specific than the Liebermann-Burchard reaction. As with the Lifschütz reaction (Bergström and Wintersteiner, 1941–42), the substances which give the characteristic color response in the Schultz test are evidently limited to the diols such as are formed from cholesterol by mild oxidation procedures. When tested under conditions closely simulating those in tissue sections, a variety of unsaturated steroids (estrone; α-estradiol; progesterone; testosterone propionate; Δ^4 androstenedione-3, 17; Δ^4 cholestenone-3; dehydroisoandrosterone; pregnenolone)[2] gave no color development which could be confused with that obtained with cholesterol. The first six in the list were non-chromogenic under these conditions.

With respect to the significance of a negative Schultz reaction, one must recognize that this test is not as sensitive as the Liebermann-Burchard reaction. When we applied the test to gelatin sections containing cholesterol emulsified with lecithin and other fats, the lower limit of sensitivity appeared to be reached when the proportion of cholesterol to other fats was about 1:10. Since in most instances of negative reactions to be mentioned below there is also a paucity of sudanophile material and in all instances lipid birefringence is negligible, it is highly probable that these tissues are truly low in cholesterol content. Strongly positive reactions, on the other hand, probably represent cholesterol (and its esters) mixed in relatively high proportion with other fats.

RESULTS

The normal corpus luteum of pregnancy. A systematic study was made of corpora lutea of pregnant rats, using two samples for each day from 1 through 5, 5 samples for day 6, and 5 at various later days through the sixteenth. Quite different from the cyclic corpus luteum, the corpus luteum of pregnancy is esentially Schultz-negative from the first day onward throughout the period investigated. Scattered cells contain clusters of medium-to-fine lipid droplets which give a yellow-brown color in the Schultz reagent. In thin sections (ca. 10 μ) no trace of blue-green is visible, although in thicker sections (ca. 30 μ) a faint background tint of this color may occur. The impression which one gains by first glance at the thin sections either in the Schultz test or after Sudan staining is that they might have been extracted with fat solvents. Most of the cells seem empty of granular fatty material.

In the fresh condition, pregnancy corpora lutea are colorless, watery in appearance by contrast with the surrounding interstitial tissue. Thick slices of such corpora pressed between slide and coverslip are pellucid in ordinary light and are but weakly illuminated under the polarizing microscope. In frozen sections no fat-soluble birefringent material is seen.

The above description also applies to corpora lutea during pseudopregnancy induced by sterile mating.

Administration of estrogen. In the first experiments with estrogen, single injections of 50 μg. estradiol benzoate were administered to 9 pregnant rats and the animals were killed approximately 48 hours

later. Arranged chronologically for days after fertilization, these cases were distributed as follows: day 4, four cases; day 5, two cases; days 10, 12, 17, one each. At autopsy it was clearly evident that the corpora lutea in all instances contained a greatly increased amount of lipid. In contrast with the pellucid tissue of normal pregnancy corpora

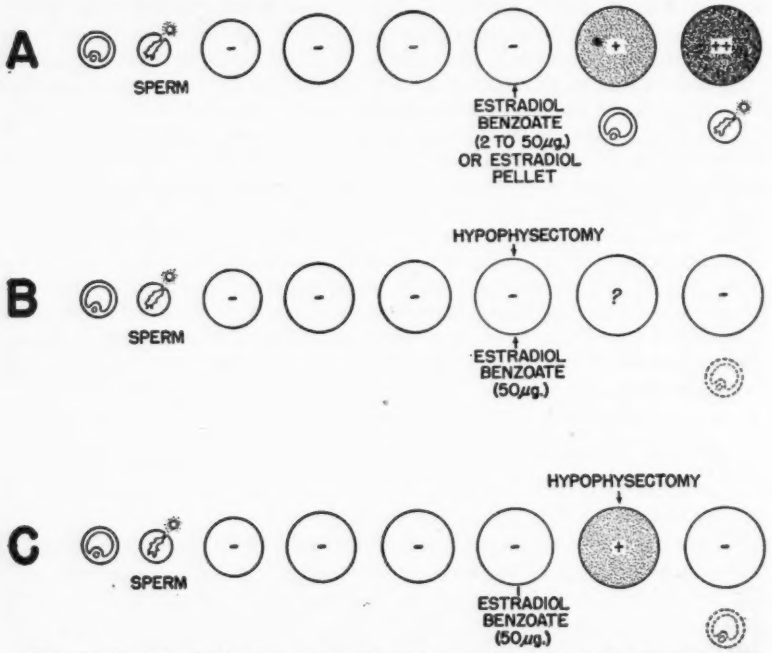

FIG. 1. Graphic illustrations of experiments showing cholesterol storage in pregnancy corpora lutea after estrogen administration (A) and absence of this effect in hypophysectomized rats (B and C). Arranged horizontally after the letter designating the type of experiment, each symbol represents one day. Thus the day of proestrus is indicated by the unruptured follicle, the following day by the newly ruptured follicle and the succeeding days of pregnancy (1–6) by circles, each representing an organized corpus luteum. In A the induction of ovulation which accompanies cholesterinization of corpora lutea is represented by the diagram of a newly ruptured follicle at the extreme right. The dotted outlines of follicles in B and C represent atresia.

lutea, these were creamy, the amount of lipid seeming to be quite comparable to that in the interstitial tissue. The texture of the fresh corpora lutea when cut with iridectomy scissors was strikingly similar to that of the adrenal cortex. Thick slices observed in the polarizing microscope were brightly illumined, equivalent or nearly so to interstitial tissue. Ovulation was demonstrated in every animal. The newly ruptured follicles were comparable to those described by Boling (1942) in normal rats 12 hours after the beginning of heat.

Frozen sections of the fatty corpora lutea obtained in these estro-

gen experiments gave a strongly positive Schultz test (++ to +++) with uniform distribution of the chromogen throughout the parenchyma, very similar to that seen in corpora lutea of the 5-day cyclic rat on the third day of diestrus (Everett, 1945, b, figs. 3, 17). This uniform distribution of lipid was also seen in the Sudan preparations, where the cytological features proved to be equally reminiscent of cyclic corpora in late diestrus. Crystalline elements were abundant (cf. Everett, 1945, b, for discussion of the relation of such crystals in fixed material to the liquid crystalline nature of lipids in the fresh tissue).

The experiment was repeated by substituting small estradiol crystals (2 cases) or estradiol-beeswax pellets (2 cases) for the oily solution of estrogen. Implantation of the crystals or pellets was effected on day 4 of pregnancy and the animals were killed on day 6 (fig. 1, A). The results were identical to those just described, both with respect to the lipid content of the corpora lutea and to the induction of ovulation. The same was true of 2 rats treated with estradiol benzoate (50 μg.) during pseudopregnancy (injected on day 6 and killed 48 hours later).

In 4 cases the corpora lutea were removed for preservation 24 hours after injection of 50 μg. estradiol benzoate on day 4. A small amount of Schultz chromogen was found in the corpus luteum sections, but the + rating given this stage in figure 1, A is rather generous. The follicles were unruptured.

In a series of 11 experiments with estradiol benzoate in oil, the dose was graded down to a minimum of 0.2 μg., injected in each case on day 4, with autopsy on day 6. The results are summarized in table 1.

TABLE 1

No. of rats	Amount estradiol benzoate injected	Ovulation	Schultz test of corpora lutea
1	25 μg.	+	++ to +++
2	10 μg.	+	++ to +++
2	3 μg.	+	++ to +++
1	2 μg.	1 ovulated foll.	++ to +++
1	2 μg.	−	++ to +++
1	1 μg.	−	+
1	1 μg.	−	−
2	0.2 μg.	−	−

The thresholds for ovulation and for corpus luteum cholesterinization are apparently of the same order of magnitude, suggesting that a common factor is responsible for both events.

The following experiments demonstrate that the cholesterinizing action of estrogen occurs only in the presence of the hypophysis and is thus probably indirect. In 3 control experiments, pregnant rats were hypophysectomized on day 4 and the corpora lutea were taken on

day 6. These were Schultz-negative, differing from the normal pregnancy corpora only by an increased amount of a brown-chromogenic lipid. Four rats were then hypophysectomized on pregnancy day 4 and immediately afterward were injected with 50 μg. estradiol benzoate (fig. 1, B). Two days later the corpora lutea were Schultz-

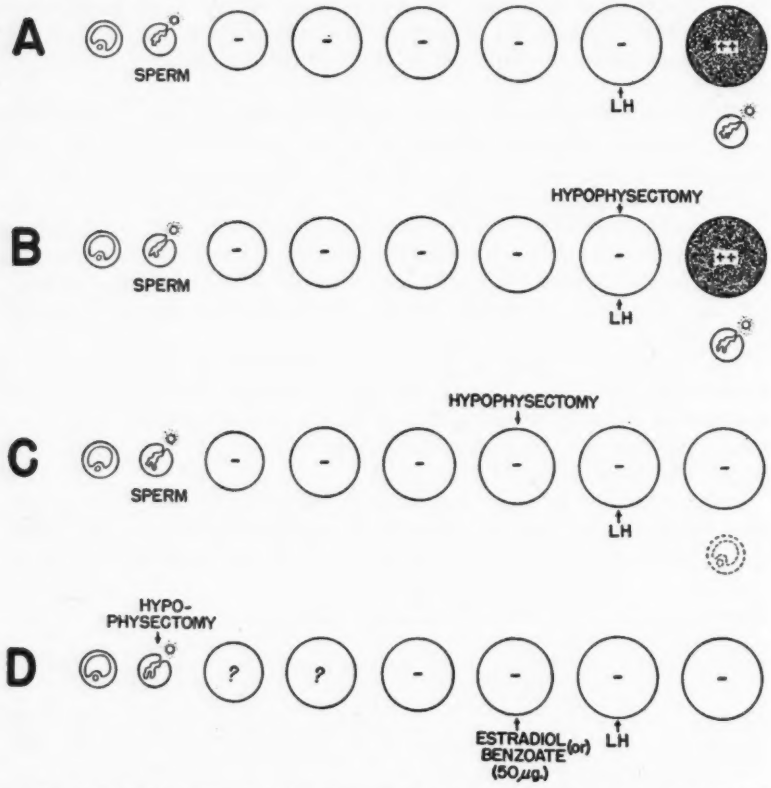

FIG. 2 A. LH induces cholesterol storage and ovulation in pregnant rats within 24 hours after injection.

B. The hypophysis need not be present.

C and D. Hypophysectomy 24 hours or more before LH injection prevents cholesterinization.

negative, closely resembling those from hypophysectomized controls. Like amounts of estradiol benzoate were injected into 3 rats on day 4 of pregnancy and the animals were hypophysectomized on day 5 (fig. 1, C). Again, the corpora lutea were Schultz-negative on day 6.

Because of this evidence of hypophyseal mediation and the previously mentioned indication that a common factor produces both the

cholesterol deposition and ovulation, it was postulated that LH is the effective agent subservient to luteotrophin.

Administration of hypophyseal LH. For the reasons just mentioned a sample of purified sheep hypophyseal LH was obtained. Eight pregnant rats were each injected with 3.3 mg. in 0.33 cc. distilled water. When autopsied 18 to 24 hours later, all animals had ovulated and all of their corpora lutea were obviously fatty. Schultz tests of the corpora were strongly positive in 7 of the 8 cases (fig. 2, A). With respect to the Schultz reaction, Sudan staining and birefringence, the corpora lutea of the 7 rats were indistinguishable from those in which cholesterinization had been induced by estrogen. The digitonin-precipitable fraction of the Schultz chromogen (representing free cholesterol) was also of the same order in both instances ($\frac{1}{3}$ to $\frac{1}{2}$).

This effect of LH was subsequently demonstrated in the absence of the hypophysis. Four pregnant females were hypophysectomized on day 5 immediately before receiving 3.3 mg. of LH (fig. 2, B). All of these ovulated within 24 hours. Cholesterol was stored in the definitive corpora lutea of pregnancy in equal degree to the last mentioned cases (++ to +++ Schultz). However, 4 other rats hypophysectomized on day 4 and injected with 3.3 mg. LH 24 hours later, failed to produce Schultz-positive corpora lutea (fig. 2, C). They appeared rather fatty at the time of removal, and the sections contained an unusually large amount of sudanophile lipid, in excess of that seen at 48 hours after hypophysectomy alone. This fat gave rise to a brown pigment in the Schultz reaction and did not form acetone-insoluble digitonides. Clearly the immediate presence of the hypophysis is not essential to cholesterinization of corpora lutea by LH, but very recent luteotrophic stimulation is required.

A short series of experiments gave an estimate of the time-lapse required for cholesterol accumulation after LH administration. From the initial experiments it was evident that at 18 hours the process had reached completion. Six additional rats on the fifth day of pregnancy were each injected with 3.3 mg. of LH at a certain hour. Two were killed at 9 hrs., 2 at 12 hrs. and 2 at 15 hrs. after injection. The corpora were Schultz-negative at 9 hrs., showed a slight positive response (±) at 12 hrs., and were rated as + at 15 hrs. In the last 2 rats ovulation had already occurred. It thus appears that the fullest degree of cholesterol storage is not reached until after ovulation, 15 to 18 hours after LH injection.

Such a single injection of LH does not seem to impair corpus luteum function. Pregnancy is not interrupted as it is following the estrogen injections. In fact, after LH treatment the storage of cholesterol is not maintained. In 4 rats injected on the fourth day of pregnancy and autopsied 48 hours later, the definitive corpora lutea of pregnancy were quite free of visible lipid, a surprisingly complete dissolution of the sterol having occurred since the 24-hour stage.

Implantation had occurred in all these instances and deciduomata were forming. The same description holds for 1 animal killed 72 hours after injection and 2 rats killed at an interval of 5 days. The implantation sites in each instance appeared normal for that particular stage of pregnancy. It is recognized, of course, that the extra set of corpora lutea induced by LH may have also become functional in these experiments.

Cholesterinization of corpora lutea while maintained by lactogen therapy. Six cyclic females were each given a series of daily injections

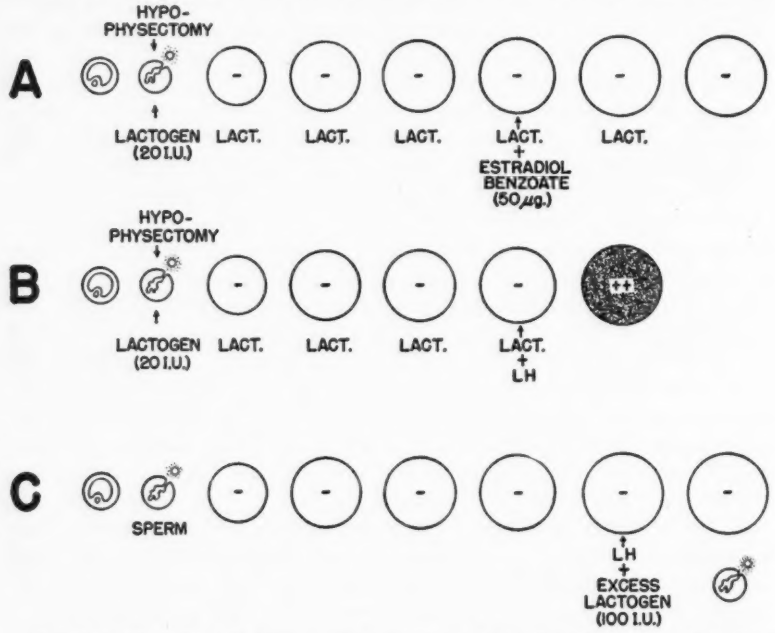

FIG. 3. A. Non-effect of estrogen on corpora lutea maintained by lactogen injections after hypophysectomy.

B. Cholesterol storage induced by LH in corpora lutea maintained by lactogen after hypophysectomy.

C. Excess of lactogen prevents cholesterol storage following LH treatment.

of 20 I.U. of lactogen, beginning in stage III of a cycle. Three of them received lactogen S and the others lactogen A. On the fourth day of leucocytic smears each animal received an injection of 50 μg. of estradiol benzoate. Two days later all rats were found to have fatty corpora lutea, the sections of which were strongly Schultz-positive (++ to +++). Parenthetically it should be noted that the animals which had been treated with the more highly purified lactogen S failed to ovulate, possibly an expression of the "anti-gonad" activity of the lactogenic hormone (Riddle and Bates, 1939). Contrariwise, all

three rats injected with lactogen A ovulated, perhaps a result of its impurity. I interpret the cholesterol storage in these instances to be the result of LH secretion evoked by estrogen in the same manner as when estrogen is injected during pregnancy or "natural" pseudopregnancy.

The above experiment was repeated in the absence of the hypophysis with negative results (fig. 3, A). Seven rats were successfully hypophysectomized in stage III of their cycles. Daily injections of 20 I.U. lactogen S were instituted at once in 5 cases. In the other two, twice-daily injections of 15 I.U. were given. All 7 rats received either 50 μg. estradiol benzoate or an implanted crystal of estradiol on the fourth day of leucocytic smears and were killed on the sixth day. The corpora lutea were Schultz-negative and almost free of visible fat in Sudan-stained preparations, thus resembling the corpora of normal pregnancy or pseudopregnancy.

An experiment of similar design to that last mentioned establishes the respective rôles of luteotrophin and LH in the cholesterol storage phenomenon. Three rats hypophysectomized a few hours after ovulation received the first of a series of daily injections of lactogen S (20 I.U.) immediately after operation. Four days later each animal was also injected with 3.3 mg. of LH. On the following day the corpora lutea were found to be "creamy." The Schultz tests were rated ++ in 2 cases and + to ++ in the other (fig. 3, B). In control experiments, 2 cyclic females hypophysectomized shortly after ovulation remained untreated for 4 days thereafter and were then injected with the standard dose of LH on the fifth day. As shown in figure 2, D, these corpora lutea were Schultz-negative 24 hours later.

Effect of excess lactogen upon the action of LH. This particular phase of the study developed from experiments in which single injections of large doses of lactogen A were given to 2 rats on the fifth day of pregnancy. As indicated previously, this preparation was known to be contaminated with LH, a significantly effective amount being present in 100 I.U. of lactogen. Doses of 100 and 300 I.U., respectively, were administered. After autopsy 24 hours later, the corpora lutea were Schultz-negative, although ovulation had been induced in both cases.

Five additional pregnant rats were each injected with 100 I.U. of lactogen A and at the same time in a different injection site were each given the standard dose of LH (3.3 mg.). These corpora lutea were similarly Schultz-negative (fig. 3, C) and contained no more sudanophile lipid than those of normal pregnancy. Ovulation was demonstrated in each case. The experiment was repeated in 4 rats, employing lactogen S (100–180 I.U.). The results were the same. It thus appears that given a certain degree of luteotrophic stimulation, a sharp increase of LH brings about cholesterol deposition, but if luteotrophic stimulation also sharply increases, sterol accumulation does

not occur. Directly pertinent to this consideration is the fact noted earlier that cholesterinization of pregnancy corpora lutea is transient (<24 hrs.) following a single injection of LH, indicating that as secretory activity continues the cholesterol store is utilized.

DISCUSSION

If one assumes, on the basis of the last-mentioned experiment, that corpus luteum cholesterinization in this species depends upon the ratio between LH and the lactogenic hormone, an explanation is offered for the sequence of changes in cholesterol content of the corpus luteum of the cyclic rat (Everett, 1945, b). Since Astwood's (1939) evidence indicates that no effective progesterone secretion occurs during diestrus in the rat, luteotrophic stimulation must be minimal during such time, but is probably not completely absent (Everett, 1944; 1945, b). Given a certain minimal degree of such stimulation, remaining fairly constant throughout the interval, the observed progressive increase of corpus luteum cholesterol can be regarded as a measure of gradual elevation of LH as diestrus advances. The LH/luteotrophin ratio thus becomes high on the final day of diestrus. Since it is probable that LH continues to increase with more rapid acceleration during proestrus, the transient depletion of corpus luteum cholesterol which I observed during "pre-proestrus" (Astwood, 1939) and proestrus must express a sharp rise in luteotrophin such that the LH/luteotrophin ratio becomes depressed. During this period progesterone is apparently secreted in physiologically effective amounts (Astwood, 1939, 1941). Renewed cholesterol storage which is seen after ovulation can be regarded as an expression of a spike in the LH curve during preovulatory swelling. In addition, the effective luteotrophin level may well have decreased by this time, resulting from its diminished secretion and (or) from its utilization by the newly-formed corpora lutea.

This leads directly to the question whether the functional life of cyclic corpora lutea may extend beyond the time of the next ovulation. Certainly there is a pronounced reduction in volume at this time (Boling, 1942) and cytochemically many of the cells show signs of fatty degeneration, but throughout even the next cycle or two considerable numbers of luteal cells seem to remain healthy. From occasional observations of such regressing corpora lutea in cyclic rats and early pregnancy, in the presence and absence of the hypophysis, the impression has been gained that Schultz-positive lipids occur in them only if the hypophysis is present. Is the onset of regressive changes the result of insufficient luteotrophin to maintain a low LH/luteotrophin ratio? Are they an expression of exhaustion of cells resulting from overstimulation during porestrus after minimal activity during the interval? Or is the high level of estrogen during proestrus a damaging factor? Estrogen can interrupt pregnancy, but to

what extent it can directly produce corpus luteum damage is not clear. It is well known that under suitable conditions prolonged corpus luteum secretion can occur in the presence of estrogen.

The mere fact of cholesterol storage in corpus luteum cells does not necessarily imply regression. We have seen above that although cholesterol is richly deposited 18–24 hours after an injection of LH during pregnancy, gestation continues and the cholesterol disappears within the following 24 hours. Even the continued presence of extensive cholesterol stores does not necessarily mean loss of function, for experiments to be reported elsewhere (Everett, unpublished) demonstrate that during pseudopregnancies resulting from continuous estrogen treatment in rats, the corpora lutea become strongly Schultz-positive long before they cease to suppress vaginal cornification.

What relationship, then, does cholesterol storage bear to progesterone secretion? Whether the sterol is locally synthesized or brought in from the blood stream cannot be known at present. Bloch, Borek and Rittenberg (1946) were of the opinion that the liver is the principal locus of cholesterol synthesis in the animal body. They found that tagged acetic acid was slowly converted to cholesterol in incubated liver slices. A limited number of other tissues was studied, including testis, with no evidence of such transformation. Neither adrenal cortex nor ovarian tissue were investigated. Even had they been included, negative results would be inconclusive, for much recent evidence indicates that cholesterol metabolism in adrenocortical and gonadal tissue is controlled to great extent by hormonal influences.

Most of that evidence to date has been concerned with the seemingly rapid utilization of cholesterol during accelerated secretion. Such a change occurs in the adrenal cortex in response to strong adrenocorticotrophic stimulation (Long, 1947, and literature cited there). Claesson and Hillarp (1946) have very recently brought forward correlated chemical and histochemical evidence that cholesterol is mobilized in the interstitial cells of the rabbit ovary within a few hours after copulation or after administration of mare serum gonadotrophin. Similar changes occur in interstitial and thecal tissue of the rat ovary, associated with spontaneous cyclic ovulation or following treatment with gonadotrophins (Everett, unpublished; Claessson and Hillarp, private communication).[4] All of these phenomena have been regarded as evidence that cholesterol is the parent material from which the specific steroid hormones of these organs are derived.

The present evidence not only aligns the corpus luteum with other steroid-secreting tissues, but also indicates that one hypophyseal hormone (LH) is specifically concerned with cholesterol accumulation in luteal cells while the luteotrophic hormone (lactogen) is to large

[4] If one assumes that the birefringent lipids described in the cyclic rat ovary by Dempsey and Bassett (1943) are largely cholesterol and its esters, their findings agree with these observations, as regards theca interna.

extent concerned with the later steps in progesterone synthesis. The inverse relationship in the sow corpus luteum between cholesterol content and progestational potency of extracts, caused Boyd and Elden (1935) to emphasize the possibility that cholesterol is utilized in formation of the corpus luteum hormone. Biochemical evidence strongly supporting this view was obtained by Bloch (1945). After feeding deuterio-cholesterol to a pregnant woman, significant amounts of deuterio-pregnandiol were recovered from her urine. There was, of course, no direct implication of the corpus luteum in that particular transformation. The experiments with excess lactogen, as described above, cause one to believe that not only is cholesterol transformed to progesterone by luteal cells, but that the rate of this transformation may be quantitatively dependent upon the level of luteotrophin. Beyond a certain point, on the other hand, the rate would appear to be independent of the rapidity of cholesterol accumulation.

If local cholesterol synthesis (or its accumulation from an extra-luteal source) is an essential initial step in progesterone formation, it follows that any agent such as lactogen which by itself is capable of maintaining corpus luteum secretion must have a certain minimal capacity to produce cholesterol. It also follows that under ordinary conditions of "full" corpus luteum activity, as in pregnancy or natural pseudopregnancy, cholesterinization is nicely balanced with the later steps of progesterone synthesis. No storage of cholesterol then occurs. If minimal cholesterol production is simply one of several attributes of a single entity, lactogen, then it would appear that LH is capable of substituting quite exclusively for this one attribute. Granting this, one might logically expect that other agents may be found which may substitute exclusively with respect to later steps in progesterone synthesis. Combined with LH such a substance might produce the full effect of maintaining corpus luteum secretion, yet by itself be ineffective.

Whatever truth exists in these considerations, the mere fact that one can at will increase or decrease the cholesterol store within luteal cells offers an entirely new approach to corpus luteum physiology.

SUMMARY

Rich deposition of cholesterol occurred in the corpora lutea of the pregnant rat 48 hours after injection of estradiol benzoate (2 μg. or more) or after implantation of small estradiol crystals. Slight accumulation was in progress 24 hours after estrogen administration, but the major change coincided, approximately, with induced ovulation (ca. 36 hours). The presence of the hypophysis was essential.

Sheep LH injected on the fifth day of pregnancy regularly caused both ovulation and cholesterol storage in corpora lutea within 18 hours, even when the hypophysis was removed at the time of injection. However, if the gland had been excised earlier (24 hours or more)

neither event transpired. In hypophysectomized rats in which corpora lutea were maintained by LH-free lactogen, injection of LH caused cholesterol accumulation, while administration of estradiol benzoate failed.

Injection of an excess of lactogenic hormone at the same time that LH was introduced prevented cholesterinization of the corpora lutea. It thus appears that whether or not cholesterol storage occurs depends upon the ratio of LH/luteotrophin. LH appears to have a specific action of increasing the cholesterol store in luteal tissue, whereas the degree of luteotrophic stimulation determines the rate at which the cholesterol is utilized in the secretory process. The observations add to the body of evidence which indicates that cholesterol is a precursor of progesterone.

A reinterpretation of the sequence of changes in the cyclic corpus luteum of the rat is presented.

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OBSERVATIONS ON THE FLUORESCENCE, BIREFRINGENCE AND HISTOCHEMISTRY OF THE HUMAN OVARY DURING THE MENSTRUAL CYCLE

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INTRODUCTION

THE LIPIDS in the human ovary have been subjected to sporadic investigations during the present century. The earlier work utilized the fat solubility of the Sudans and the reactions between fat and osmium tetroxide. In addition, birefringence studies have been carried out, as well as chemical analyses. Chydenius (1926) concluded that the fresh human corpus luteum contains only a small amount of material which stains with Sudan III or osmium tetroxide, until degeneration of the corpus luteum occurs, with a great increase in fat at this time. Moulon (1912) noted that in the early stages, in several species including man, the corpus luteum contains doubly refractile droplets. From his description, it appears that the cells containing the anisotropic materials were chiefly or entirely those of the theca interna. Weinhouse and Brewer (1942) correlated by chemical means the various lipid fractions of the corpus luteum with the stages of its development. These investigators showed that free cholesterol remained relatively constant in the corpus luteum throughout the menstrual cycle. Cholesterol esters declined slightly with increasing activity, reaching the lowest values in the actively functioning corpus luteum of pregnancy. During the phase of regression, cholesterol esters showed a five-fold increase. In contrast, phospholipid concentration varied directly with the stages of activity, showing maximal values during maximum function. The glycerides were greatly increased during regression.

Interest in the ovarian lipids has been greatly stimulated by the discovery that the ovarian hormones are fat soluble sterols. Recently, in an effort to localize and identify the ovarian lipids in tissue sections, histochemical and other special techniques have been utilized in addition to the previously used dyes (Dempsey and Bassett 1943, Everett 1945). These methods were applied in the study of the rat ovary. The present study was undertaken in the hope of localizing the lipids, particularly the steroids, in the human ovary, and noting the changes that occurred during the menstrual cycle, with the ultimate possibility

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of correlating cellular morphology and histochemistry with ovarian physiology.

MATERIALS AND METHODS

A series of 29 ovaries was collected at the time of operative removal from women in the reproductive era who were undergoing oophorectomy and hysterectomy. It was necessary to have a sample of endometrium in each of these cases in order to ascertain the date of the menstrual cycle and therefore the age of the corpus luteum. The method used for dating the endometrium was that described by Hertig (1945). As a further check on the age of the corpus luteum, the morphologic changes in the aging corpus luteum, as described by Brewer (1942), were utilized in dating each specimen. Two to three corpora lutea of the same age from different patients were studied, corpora lutea dated at 15, 17, 18, 19, 22, 23, 24, 25, and 27 days, plus early menstrual and late menstrual, were available.

The ovaries were bisected and the half to be used for special studies was fixed in a solution of ten per cent formalin for 24 hours or longer. Serial sections were then cut on the freezing microtome at 15–50 micra thicknesses. The 15 micra sections were stained with Sudan IV. For studies of fluorescence and birefringence, 50 micra sections were mounted on slides in non-fluorescent glycerine, and observations were made with a Spencer polarizing microscope, using plane-polarized light, and a fluorescent microscope, using a beam of rays obtained by filtering the light of a carbon-arc lamp through a copper sulfate solution and a Corex filter #586. Alternate sections, 50 micra in thickness, were placed in acetone for 24 hours, and then mounted in glycerine and subjected to these examinations.

The phenylhydrazine method of Bennett (1940) was used on 50 micra sections. Alternate sections were extracted with acetone for 24 hours and then submitted to the phenylhydrazine reaction.

Fifty micra sections were also treated with concentrated sulfuric acid. The sections were mounted on slides and the excess water was blotted off. A drop of concentrated sulfuric acid was placed on the tissue and a coverslip dropped on immediately. Sections extracted with acetone for 24 hours were similarly treated.

SIGNIFICANCE OF SPECIAL TECHNIQUES

Sudan IV

Lipid droplets can be identified in frozen sections by the fact that they take up the Sudan dyes. The depth of color of the droplets with the Sudan stain cannot be related to the chemical constitution of the fats, but is attributable to the factor of physical solubility (Lison, 1936). The Sudans are soluble in neutral fats, fatty acids, cholesterol esters and other lipids. (Kaufmann and Lehmann, 1926). It is well known that lower members of the Sudan series do not stain all lipids, since they are relatively insoluble in such lipoidal substances as the phospholipids and cholesterol (Dempsey and Wislocki, 1946), and will not detect lipids bound with protein.

As will be pointed out subsequently, the size of the sudanophilic droplets in the corpus luteum varies at different stages of development. It is of interest to note, in this connection, that in the adrenal gland several investigators (Selye 1937, Sarason 1943, and Dalton et al. 1944) have described the fat

droplets as being small when the gland is in a state of activity, and much coarser and larger during inactivity.

Birefringence

When observed with polarized light, sterol substances appear light in a dark background. This property, which is caused by the radial symmetry of the steroid molecules in liquid spherocrystals, is an expression therefore, of the molecular pattern assumed by this class of compounds (Lison, 1936). This reaction is not specific. Cholesterol, its derivatives and their esters, frequently form birefringent crystals, as do the ovarian steroids. Although precise conclusions cannot be drawn from birefringence alone, the results from this technique, in conjunction with the results from other techniques, can yield information of considerable value.

Dempsey and Bassett (1943) described two varieties of birefringence in the rat ovary. One was a fibrillar variety, insoluble in acetone and associated with connective tissue. The other was a crystalline birefringence, soluble in acetone and present in parenchymatous cells. These two types were present in the human ovary. The observations herein reported deal only with the latter variety of birefringence.

Also, of interest in relation to birefringence is the observation that fine particles characterize active secretory function, whereas coarser, larger particles indicate inactivity or storage (Deamsly 1931, Deane and Shaw 1947, Weaver and Nelson 1943).

Autofluorescence

Purified estrogenic sterols exhibit a greenish yellow fluorescence (Bierry and Gouzon, 1936). In the rat ovary, Dempsey and Bassett (1943) found an acetone soluble, greenish autofluorescence in the theca interna of the developing follicle, while the granulosa layer gave a blue white color. By the use of the Zeiss microspectroscope, they found that this greenish fluorescence lay within the 5700 to 6700 Angstrom unit range, which is the range reported for estrogenic substances by Bierry and Gouzon (1936).

Phenylhydrazine

This substance reacts with the ketone or carbonyl group of the reactive molecules to produce a yellow phenylhydrazone. The ketosteroids have carbonyl groups. Bennett (1940) used this reaction in the study of the adrenal gland, and concluded that the reacting substance was a ketosteroid, probably the adrenal cortical hormone. However, Gomori (1942) criticized the work, pointing out that the phenylhydrazine reaction was akin to the plasmal reaction of Feulgen and Voit (1924), presumably a test indicating the presence of aldehydes of fatty acids. Dempsey and Wislocki (1946) conclude that this reaction is not capable of differentiating between aldehydes and ketones, and offer convincing evidence in favor of this view.

Sulfuric Acid Reaction

The addition of this substance to tissue results in the production of colored reaction products, in the presence of various unsaturated polycyclic compounds. Following the addition of acid to reacting tissue, reddish-brown droplets or granules developed within 60 seconds. In essence this is the Lie-

bermann-Burchardt reaction applied to tissues. Although originally regarded as a specific for cholesterol (Romeis, 1928), other compounds related to cholesterol, including many of the biologically active steroid hormones, react positively with sulfuric acid (Schoenheimer and Evans 1936, Reichstein and Shoppee 1943). It has been shown that steroids which possess unsaturated linkages will give the chromogenic reaction (Fieser, 1937).

Solubility in Acetone

This test has a negative value. Since the ovarian hormones are soluble in fat solvents, any substance which, after extraction with acetone, still reacts with the above reagents, should probably not be regarded as a functioning ovarian steroid.

In summary, any substance which has all these properties will be a polycylic molecule with a double bond somewhere in the molecule; will have a carbonyl group attached to it; and will be dissolved by fat solvents. The ketosteroids have such a structure and give these reactions. It is clear that no one of the reactions is specific, but taken together, they are very informative.

RESULTS

Sudan IV

With the use of Sudan IV, sudanophilic material in the developing follicles was confined to the theca interna layer. None was present in the granulosa cells nor in the surrounding connective tissue of the ovary.

In the 15 day corpus luteum, sudanophilic granules were seen throughout all the granulosa lutein cells, in fairly large numbers; an abrupt change from the appearance of the granulosa layer of the unruptured follicle. Equally large amounts were seen in the theca lutein layer. During the ensuing 6–7 day period, very fine small sudanophilic granules were seen in almost every granulosa lutein cell. These granules were present around the periphery of each cell and formed a thin red ring, lying just under the cytoplasmic membrane. In the theca lutein layer during this same period, there was a steady increase in the number and size of sudanophilic droplets. The theca became more and more conspicuous. The droplets in this layer during this period, were much larger than those in the granulosa lutein cells. At approximately the 23rd day, there was a marked decrease in the sudanophilic material of the granulosa lutein layer, many cells being devoid of the substance. Patchy areas, containing large droplets, were seen in the granulosa lutein layer around the blood vessels of the invaginating connective tissue septae and were present in large patches in the now prominent theca layer. This apparent decrease in sudanophilic substance was maintained in the granulosa lutein cells until the 27th day. About the 24th day, there was also a decrease in the number of droplets in the theca lutein layer. On the 27th day, the granulosa and theca layers were practically indistinguishable from each other on the basis of the fat stain. They both were filled with

large numbers of large red droplets. This greatly increased amount was present throughout the period of menstruation. Second generation corpora lutea also presented a complete filling of every cell with large sudanophilic droplets. These findings are in substantial agreement with Dr. Hertig's (1941) observations in this laboratory for the past several years.

Birefringence

No doubly refractile material was found in or around the primordial ovum, the primordial follicle or the small primary follicle. Birefringence first appeared as small, scattered crystals in the theca interna in the small follicles at the stage of antrum formation (Fig. 1 and 2). The number of crystals increased as the follicle developed and were always confined to the theca interna layer. They were never seen in the granulosa cell layer (Fig. 3 and 4). In the largest follicles, which were either atretic or preovulatory, there was a dimunition in the number of crystals with a thin, patchy rim of crystals in the theca interna.

Following ovulation a very few tiny birefringent crystals were noted in the theca lutein layer of the 15 day corpus luteum (Fig. 5). There was a steady increase in the number and size of the crystals in the theca layer up to the 24th day. Very fine crystals first appeared in the granulosa lutein layer on about the 17th day (Fig. 6). These were present in slightly increasing numbers until the 22nd day (Fig. 8). During this period, 17–23 days, there was a consistently larger amount of crystalline material in the theca lutein layer than in the granulosa lutein layer.

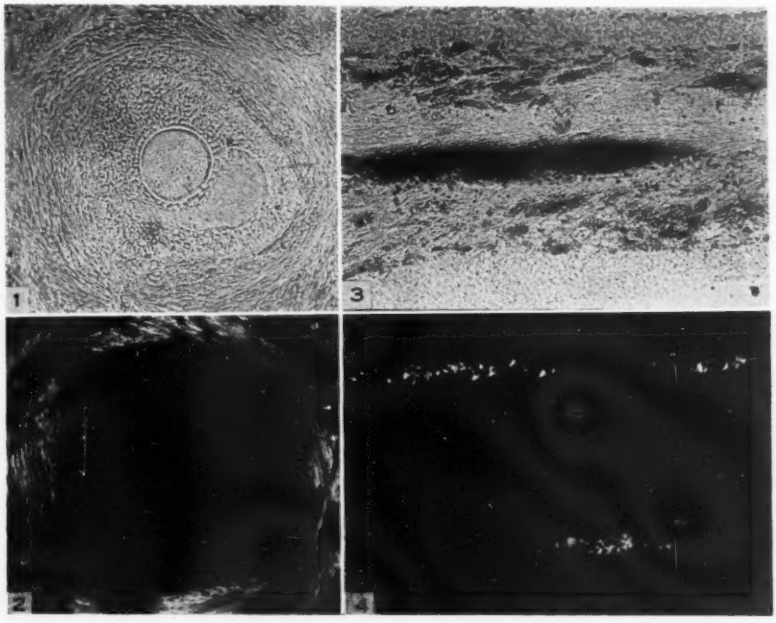
There was a decided diminution in the amount of crystalline material following the 22nd day (Fig. 9). Most of the specimens showed a complete absence of crystals in the granulosa lutein cells during the period from the 23rd day to late menstruation. The 27 day specimen was the one exception to this rule. Finally, in the corpus luteum of late menstruation, the granulosa lutein cells again contained moderate numbers of crystals.

At approximately the 24th day, there was a noticeable decrease in the birefringent material in the theca lutein layer (Fig. 9). The decrease in the theca lutein layer was maintained until the 27th day. Following this there was a considerable increase in large birefringent crystals during the menstrual period. The material assumed a dense patchy pattern (Fig. 10). The second generation corpora lutea were completely filled with large patches of crystals. Birefringence was also present in the interstitial cells of the ovarian stroma. Acetone completely extracted the crystals in the parenchymatous cells.

Autofluorescence

The theca interna of the developing follicles emitted a light yellow-green fluorescence which stood out in sharp contrast to the dark

blue color of the stroma and to the light blue color of the granulosa cells. With one exception, the pattern of yellow-green fluorescence followed that of the birefringence studies. This exception was the 15 day corpus luteum, in which the yellow-green fluorescence was present in the granulosa layer in large amount, as well as in the theca layer. All the other tests were negative in the granulosa lutein layer of this corpus luteum. This fluorescence was not due to Vitamin A, since it did not fade on long exposure to ultraviolet light.



FIGS. 1 and 2. A small follicle at the stage of early antrum formation. Figure 1 is a photograph of an unstained frozen section taken with ordinary light, while figure 2 is the same section photographed between crossed Nicol prisms. A few tiny birefringent crystals may be seen in the developing theca. These are not as prominent in this photograph as under direct microscopy. The streaks of birefringence arranged in whorls around the follicle represent connective tissue bands and are not acetone soluble. Note absence of crystals in granulosa layer. ×200.

FIGS. 3 and 4. Photographs made with ordinary and polarized light of two adjacent, medium sized follicles. Crystals are absent in the granulosa layer and present in moderate numbers in the theca interna of both follicles. ×200.

In the corpus luteum there were two types of yellow-green fluorescence. One was a diffuse yellowish color occurring most prominently near the inner border of the coagulum, and this substance was not acetone soluble. The other type was a patchy yellow-green material scattered in the cells, and was acetone soluble. Some of the interstital

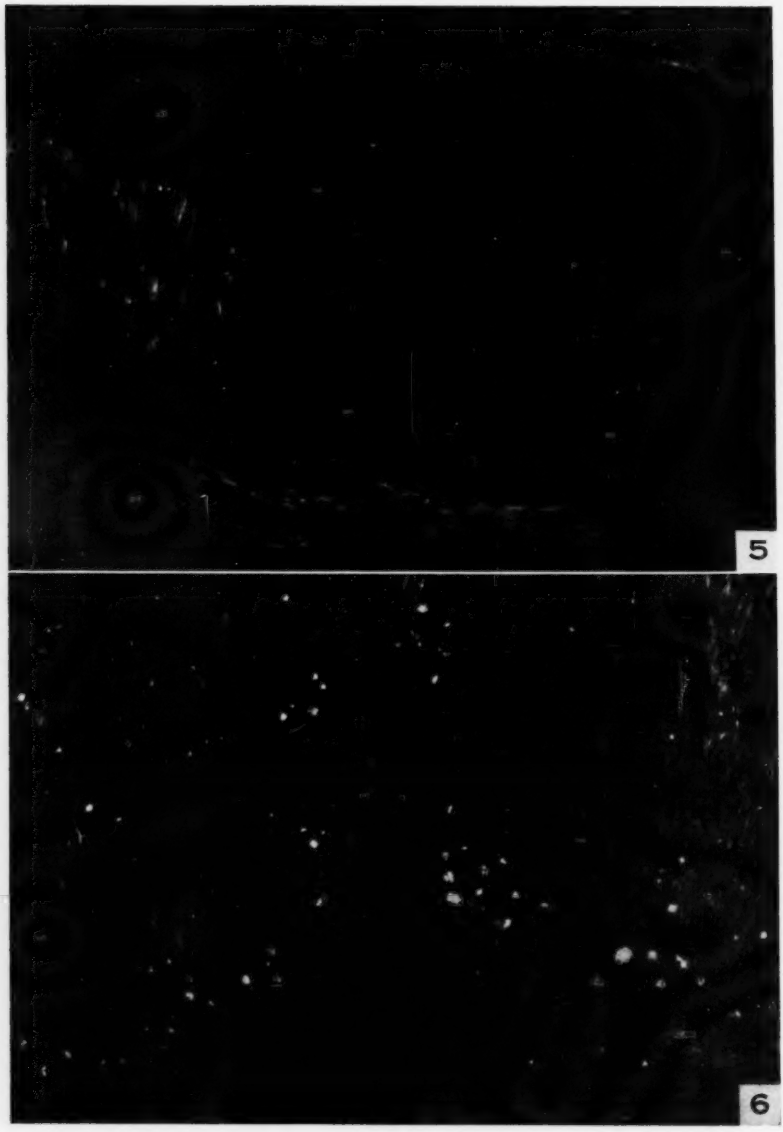


FIG. 5. Photograph of a 15 day corpus luteum made with polarized light. The theca lutein layer is filled with extravasated blood and therefore appears thicker than usual. A few small crystals are present in the theca lutein layer but are absent in the granulosa lutein layer. ×200.

FIG. 6. Photograph made with polarized light of the edge of a 17 day corpus luteum. Note increase over 15 day corpus luteum in the number of crystals in theca lutein layer at the periphery and along the invaginating septum. A few crystals are present in the granulosa lutein layer. ×200.

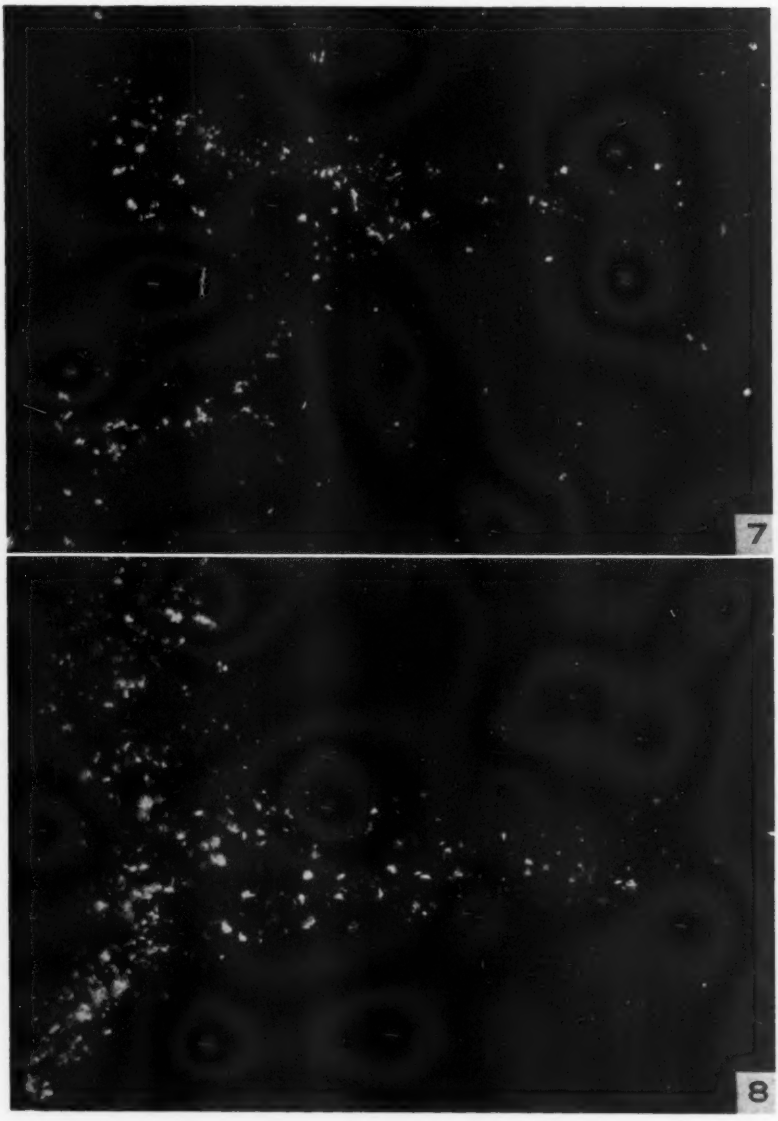


FIG. 7. Photograph made with polarized light of edge of 19 day corpus luteum, showing increased numbers of crystals in theca lutein layer. ×200.

FIG. 8. Photograph made with polarized light of edge of 22 day corpus luteum, showing large amount of crystalline material in theca lutein layer and abscence in granulosa lutein layer. ×200.

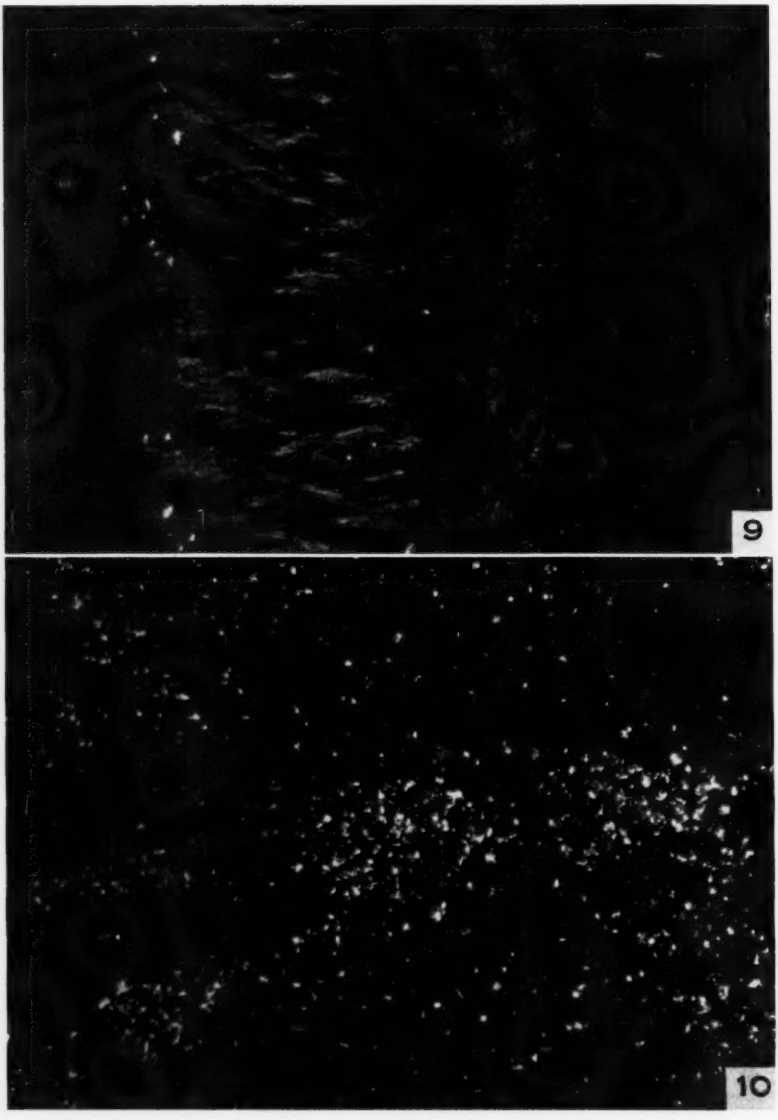


FIG. 9. Photograph made with polarized light of edge of 25 day corpus luteum, showing marked decrease in crystalline material in theca lutein layer and abscence in the granulosa lutein layer. The white streaks present in the granulosa lutein layer are associated with connective tissue. ×200.

FIG. 10. Photograph made with polarized light of edge of corpus luteum of late menstruation, showing large number of crystals in theca and granulosa lutein layers. ×200.

cells gave a brick red to yellow-orange fluorescence which was not acetone soluble. However, the majority showed a yellow-green fluorescence which was acetone soluble.

Phenylhydrazine Reaction

Yellow phenylhydrazone droplets were present in the theca interna of the developing follicle and were never seen in the granulosa cells or in the surrounding connective tissue.

Although no yellow droplets were seen in the 15 day corpus luteum, the presence of a large amount of blood in the theca layer undoubtedly masked the small amount probably present. They first appeared in the 17 day corpus luteum where they were found in the same number and location as the birefringent crystals. Throughout the cycle the phenylhydrazine reaction paralleled the results of birefringence. In addition, it was noted that the yellow phenylhydrazones were present in the peripheral region of the cytoplasm in the granulosa lutein cells until the 22nd day. This closely resembled the picture of the Sudan IV stained sections. This "peripheral rim" was not present in the granulosa lutein cells after the 22nd day, but from that day on, large extracellular oil droplets were seen in increasing numbers in the granulosa lutein layer. Some of these droplets reacted to give yellow phenylhydrazones and some did not. The reactive substances were acetone soluble.

Sulfuric Acid Reaction

Reddish-brown granules were seen in the same locations as the birefringent crystals in the developing follicle (Fig. 11).

The observations on the sulfuric acid reaction were not made in a serial fashion, so that very little can be said regarding relative amounts of reactive substances with the aging of the corpus luteum. However, throughout the whole life of the corpus luteum, the red-brown granules appeared in the same location and in roughly the same quantity as the birefringent crystals (Figs. 12 and 13). They were present in large quantity in second generation corpora lutea and in the interstitial cells of the ovary. A point worth noting is the fact that large oily red-brown droplets started to appear after the 23rd day in the granulosa lutein layer. These were present in large numbers in the corpus luteum of menstruation and in second generation corpora lutea. No reactive substances were found in sections previously extracted with acetone.

DISCUSSION

Nature of Reactive Substances

It is not possible to state unequivocally that the acetone soluble materials, reacting with phenylhydrazine and sulfuric acid, and exhibiting birefringence and yellow-green fluorescence, are the active

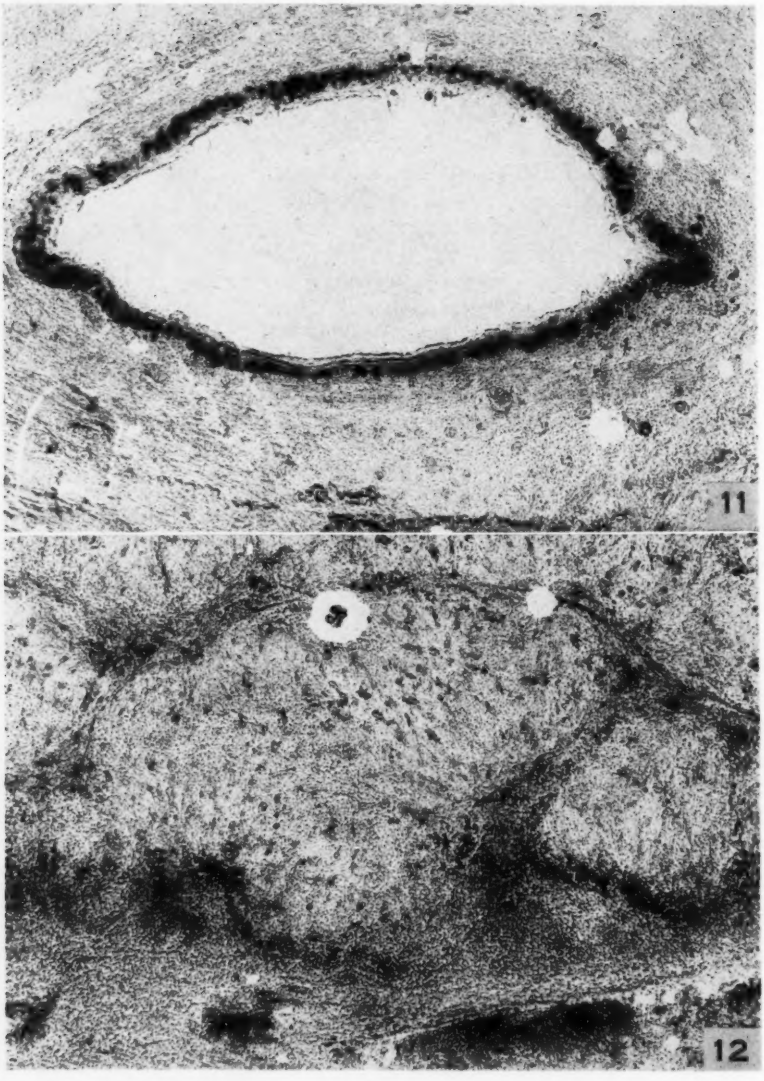


FIG. 11. Photograph taken with ordinary light of frozen section treated with sulfuric acid. Dark chromogenic material is present in theca interna and not in granulosa cells. ×100.

FIG. 12. Photograph of frozen section of 22 day corpus luteum treated with sulfuric acid, showing concentration of dark granules in theca lutein layer. ×100.

steroid hormones of the ovary. However, coupled with other known facts, it seems likely that most of these materials are the active hormones, at least with respect to the developing follicle and the active corpus luteum. For example, when one combines the two facts 1) that the greatest concentration of estrogens in the follicle is extractable from the theca cells (Corner, 1938) and 2) that upon histochemical examination the positively reacting materials are confined to the theca layer, one has strong presumptive evidence that the reactive

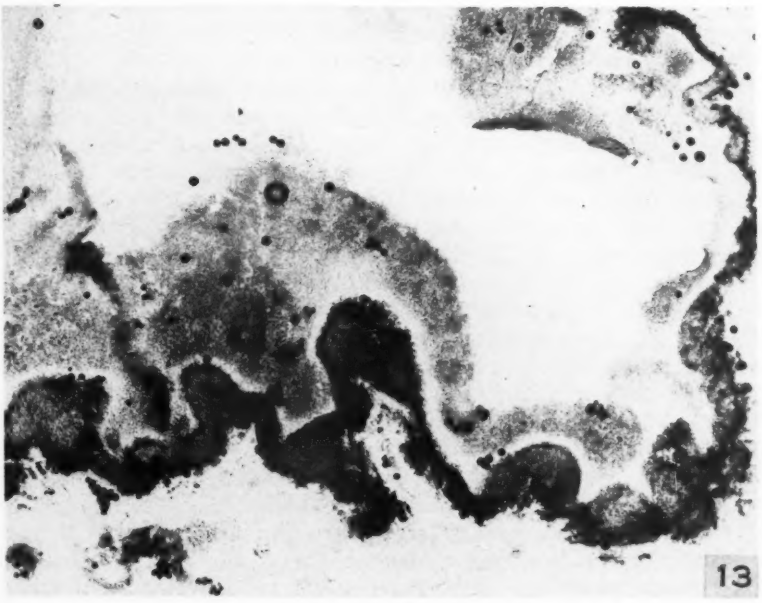


FIG. 13. Photograph of frozen section of corpus luteum of late menstruation treated with sulfuric acid, showing both theca and granulosa lutein layers filled with red brown droplets. ×50.

substances are the biologically active steroids or closely related substances. Also, since progesterone is extractable from the corpus luteum, and since substances showing reactions and properties that are exhibited by progesterone are present histologically, it is not illogical to conclude that the reactive substance is the steroid hormone itself. Furthermore, the quantitative variations in the estimated amounts of reactive substances are in agreement with the known quantitative variations of the ovarian hormones excreted in the urine (Fig. 14).

Dempsey and Bassett (1943) concluded that the reactive substances in the tissues of the rat ovary were the biologically active

steroids or closely related substances. More importantly, they concluded that tissues which did not show any of these reactions did not contain the steroid hormones.

Nevertheless, the fact remains that these reactions reveal a family of related compounds, but do not segregate the various members of this family. In some instances they may represent degradation products of ovarian steroid metabolism.

Physiological Correlation

Application of the various tests, all dependent upon different chemical and physical properties, revealed a close correspondence with respect to both location and quantity.

Reactive material, i.e., substances showing anisotropicity, greenish yellow fluorescence, yellow phenylhydrazones, and a chromogenic reaction with sulfuric acid, were never seen in the granulosa cells of the follicle. It is probably safe to conclude from this that the granulosa cells do not produce the estrogenic hormones. On the other hand, reactive substances were found in the theca interna layer of the follicle, suggesting that the theca interna layer is the site of estrogen production, an idea supported by Corner's (1938) demonstration that extractable estrogens are concentrated in the theca interna. These findings are identical with those in the follicle of the rat.

After their first appearance in the theca of the small follicles at the stage of antrum formation, the reactive substances steadily increased in amount as the follicle enlarged. However, the largest follicles, one centimeter or more in diameter, showed a decided decrease. Even though it was not possible to differentiate preovulatory follicles from follicles undergoing early atresia, it is noteworthy that all follicles one centimeter or larger had a diminished amount of reactive substance. It is interesting to compare this quantitative variation with the rise of estrogenic substances in the urine during the first half of the menstrual cycle, which is followed by a drop at the time of ovulation (Smith and Smith, unpublished data). This chart represents the measured amounts, in gamma, of α estradiol plus estrone plus estriol in the urine and therefore is indicative of ovarian secretory activity.

In the corpus luteum, sudanophilic substances and reactive materials were always found in the theca lutein layer. In the 15 day corpus luteum they were present in very small amounts. Subsequently, the amounts increased steadily to the maximum at 23 days and then diminished rapidly until the 27th day, when a marked increase occurred. The granulosa cells presented several interesting changes. In general, reactive substances were present in scant amount from the 17th day to the 22nd day; were absent from the 23rd day until late menstruation and then reappeared in the corpus luteum of late menstruation. Although an exact determination of the changing

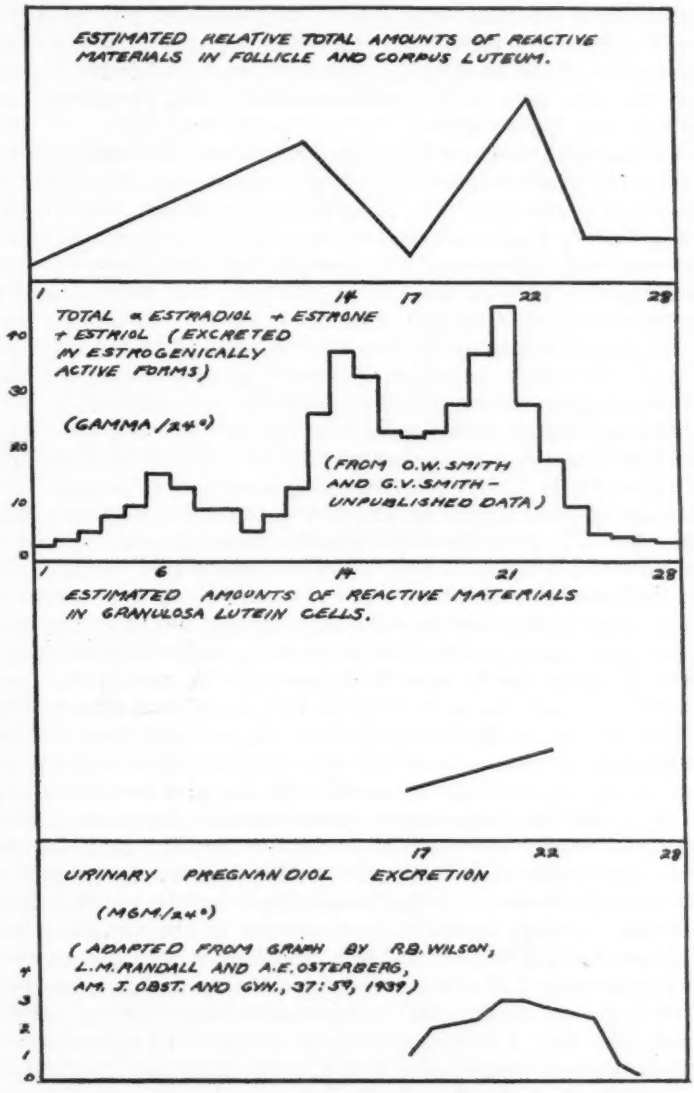


FIG. 14

total amounts of reactive materials in the follicle and corpus luteum was not possible, the changes were striking enough to permit a rough estimation. This estimation of the changing amounts during the cycle is presented graphically (Fig. 14A). There was a gradual increase in reactive material in the enlarging follicle until it reached the size of

a preovulatory follicle, with a distinct decrease at this time. In the very young corpus luteum there was a scant amount, which steadily increased until the 22nd day. A definite and marked decrease occurred after the 22nd day. In the granulosa lutein cells, the change on the 22nd day was quite striking.

Comparing this chart with the levels of urinary estrogens, as determined by Smith and Smith, and presented in Fig. 14B, one finds an interesting correlation. The total urinary estrogens rise during the first half of the cycle until the 14th day, when a drop occurs. The titer then rises still higher until the 21st day and then drops off rapidly, reaching a low level on the 24th or 25th day. The only point of disagreement in these two graphs is on the sixth day, when a peak occurs in the excretion and no similar peak is present on the estimate of amounts of reactive material. However, the elevations and depressions throughout the rest of the cycle are in close agreement.

The variation in pregnandiol excretion also fits well with the estimated amounts of reactive material in the corpus luteum. According to Wilson, et al. (1939) (Fig. 14), pregnandiol first appears on the 16th day, reaches a peak on the 21st or 22nd day and then rapidly diminishes. The peak for all these substances is reached at about the 22nd day and is followed by a rapid decline. In spite of this correlation, the possibility exists that an increase in reactive materials need not be simultaneous with an increased secretory activity. The reverse may be true, namely, that the less reactive materials present in the tissues, the more that is being discharged into the circulation.

Chemical analyses have revealed the presence of both estrogens and progesterone in the corpus luteum. Theoretically, since the corpus luteum contains two varieties of cells, the theca lutein and the granulosa lutein, it is possible to ascribe the estrogen formation to one cell type and the progesterone production to the other. Dubreuil (1940), on purely speculative grounds, concluded that the theca lutein cells produced the estrogens, while the granulosa lutein cells produced progesterone. In this connection it will be recalled that, in our study, reactive materials were present in the granulosa lutein cells from the 17th to the 22nd day. Likewise, it will be remembered that progestational effects on the endometrium and pregnandiol excretion begin on the 16th day; pregnandiol being excreted until the 24th or 25th day. This correspondence between the appearance and disappearance of pregnandiol in the urine might be interpreted as meaning that the granulosa lutein cells are the ones which produce progesterone leaving the theca lutein cells to produce estrogens.

However, it is unnecessary to invoke a doctrine of unit function per unit cell type. In the adrenal gland many hormonal fractions have been separated, and all of these are apparently elaborated by a few cell types. In the placenta, evidence has accrued that the syncytium elaborates both estrogens and progesterone (Dempsey and Wislocki,

1946). Accordingly, it may be that in the corpus luteum the theca lutein and granulosa lutein cells each elaborate both hormones. It should be noted that the appearance of pregnandiol in the urine 2 days after the formation of the corpus luteum does not mean that progesterone may not be secreted prior to this time. It is possible that the concentration in the blood stream merely does not reach a sufficient level to be excreted in the urine until the 16th day.

It is worth while to single out the changes noted after staining with Sudan IV, and with the use of phenylhydrazine, and sulfuric acid. In the granulosa lutein layer during the period from 17–22 days, these tests revealed fine droplets of reactive material in every cell, in the peripheral region of the cytoplasm, just beneath the cytoplasmic membrane. After the 22nd day, this distribution did not obtain and most of the cells did not contain reactive substances until late in the menstrual period. However, with these three types of examinations, during the period from the 22nd day until late menstruation, a few extracellular large oily droplets were seen in the granulosa lutein layer. This change from small to large droplets with the disappearance of reactive materials from many of the granulosa lutein cells, may be interpreted functionally as a change from active secretion to storage or inactivity. This interpretation is based on the finding on the adrenal gland (Selye 1937, Sarason 1943, Dalton et al. 1944) that fine lipid droplets are associated with active secretion and large droplets with inactivity. If this interpretation is correct, it offers further evidence of the cessation of the active secretion of the corpus luteum on the 22nd day.

Finally, the question arises whether the reactive materials seen in large amounts in the corpus luteum during the latter part of menstruation, in the second generation corpora lutea and in the interstitial cells of the ovary, are identical with those seen in the active corpus luteum. Everett (1945), working with the rat ovary, concluded that the increase in reactive material in the second generation corpora lutea reflected renewed function and activity. It is possible that this may be the case in the human ovary, but it seems more likely that these are degradation products which are collected in a degenerate structure, and may be cholesterol esters or other steroid materials, rather than the biologically active steroids.

SUMMARY

Sections of human ovaries, removed at different stages in the menstrual cycle, were examined in order to determine the localization of acetone soluble birefringent crystals, autofluorescent material, phenylhydrazones, and sterol substances which react with sulfuric acid. Substances giving these reactions were found in the theca interna of the developing follicle but not in the granulosa layer. They were present in the granulosa lutein and theca leutein cells of the corpus

luteum. The amounts of reactive materials varied during the menstrual cycle and these variations correspond with the variation in estrogen and progesterone excretion during the cycle.

ACKNOWLEDGMENTS

We wish to express our appreciation to Dr. G. V. Smith for suggesting this study and for allowing us to use some of his unpublished work; to Drs. E. W. Dempsey and A. T. Hertig for kind assistance in conducting the study; and to Leo Goodman of the Mallory Institute of Pathology for the photographs.

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INHIBITION OF OVULATION IN THE RABBIT BY THE ADRENERGIC-BLOCKING AGENT DIBENAMINE

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INTRODUCTION

IN PREVIOUS papers (Markee, Sawyer and Hollinshead, 1946, 1947 a, b) we have reported the results of investigations of the mechanism by which the central nervous system exerts its control over the release of luteinizing hormone in the rabbit. Using unipolar and bipolar electrical stimulation of known voltage and current we found that direct stimulation of the hypophysis by a unipolar electrode was effective in producing ovulation in the rabbit only with relatively strong currents, which gave signs of considerable spread to the central nervous system. On the other hand, localized bipolar stimulation of the hypothalamic region with a current which was ineffective when applied by the same electrode directly to the hypophysis, produced ovulation in three out of four animals. From these results we concluded that the pathway of excitation from the hypothalamus to the anterior lobe of the hypophysis was probably not through the hypothalamico-hypophyseal nerve tract, even though its fibers have been described as ending in the anterior lobe (Brooks and Gersh, 1941; Truscott, 1944) as well as in the posterior. Rather, we believed our evidence was in favor of the existence of a humoral link in the pathway between hypothalamus and anterior lobe—a humoral link considerably longer than that usually involved between nerve endings and effector organs.

Subsequently, we attempted to determine if either of the known neurohumoral agents, acetylcholine or adrenaline, might be effective in producing ovulation in estrus rabbits if applied directly to the anterior pituitary gland. While Taubenhaus and Soskin (1941) have reported that the application of acetylcholine to the hypophysis of the rat results in the production of pseudopregnancy—i.e. in the release of luteotrophic substance (lactogenic hormone) from the anterior hypophysis—we found instillation of acetylcholine into the anterior hypophysis of the rabbit to have no patent effect in inducing release of luteinizing hormone, upon which ovulation in this species depends. However, the introduction of adrenaline into the hypophysis was followed by ovulation in a significant number of cases (with the most

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favorable dilution, 1/1000 five out of ten animals receiving it ovulated). Since adrenaline so applied was found to be effective in producing ovulation, this finding was interpreted as indicating that an adrenergic mechanism is involved in the anterior lobe stimulation produced by coitus, or by other forms of sexual stimulation.

In the present paper we have attempted to secure additional evidence as to the adrenergic nature of the proposed humoral link, in so far as the release of luteinizing hormone is concerned, by the use of Dibenamine (N, N-dibenzyl-B-chloroethylamine) which Nickerson and Goodman (1947) have shown to be one of the more effective of the new adrenergic blocking agents, and by the use of Priscol (benzylimedazoline hydrochloride) which also has certain adrenolytic and sympatholytic actions (Chess and Yonkman, 1946), but is ineffective in counteracting several effects of adrenaline (Ahlquist et al., 1947). Since our results indicate that the ovulation normally following coitus in the rabbit can be prevented by the prompt injection of Dibenamine, we have also attempted to estimate the time which must elapse following coitus, in order that hypophyseal stimulation may be effective. Fee and Parkes (1929) have shown that the hypophysis must remain *in situ* for approximately an hour, in order that sufficient luteinizing hormone to produce ovulation may be released; our results with Dibenamine indicate that the actual stimultion of the hypophysis is effective within about one minute after the termination of coitus.

MATERIALS AND METHODS

Forty mature female estrus rabbits were employed in the present study; 37 of them weighed in excess of 2.5 kg., and the averge weight was 3.3 kg. Most of them had been isolated for months; a few had been kept apart only about a week, but in these the willingness to mate was indicative of nonpregnancy.

Solutions of the drugs were made on the days on which they were to be used. Dibenamine was dissolved in absolute alcohol (100 mg. per ml.) and the total dose per animal diluted to 10 ml. with unbuffered Ringer-Locke solution. Priscol was dissolved in distilled water (200 mg. per ml.) and the dose also adjusted to 10 ml. with the salt solution.

An attempt was made at first to inject the drugs prior to copulation, but it was found that the females would not accept the males after receiving effective doses of Dibenamine or equivalent ones of Priscol. The procedure adopted, therefore, was to inject 10 ml. doses into the marginal ear vein as soon as possible, or at definite time intervals, after observed mating. Before the female was placed in the cage with the male, her ear was prepared and the desired dose of drug was made ready in the 10 ml. syringe. As soon as the male fell off at the termination of coitus, the female was taken from the cage; the drug was then either injected immediately—that is, injection was begun in less than one minute—or after a wait of 3, 5, or 12 minutes. The doses of Dibenmaine employed varied from 14 to 30 mg./kg., those of Priscol from 10 to 52 mg./kg. The injection was at the rate of about 10 ml. per minute.

Following injection, the rabbits which survived were replaced in their isolation cages for a period of two days, at the end of which time their ovaries were examined at laparotomy for the presence of ruptured follicles.

RESULTS

The lower doses (14–17 mg/kg.) of Dibenamine were too small to be effective, as judged both by their failure to prevent ovulation and by the lack of physical signs resulting from the injection. The three animals receiving Dibenamine in this dose range (Table 1) all ovulated as a result of coitus.

The higher doses of Dibenamine (25–32 mg./kg.), on the other hand, always markedly constricted the pupils. Other symptoms of these doses varied from no noticeable effects to an extreme extensor rigidity, with the head pulled back and the forelegs held stiffly extended, alternating with apparently spontaneous limb movements by which they threw themselves about the cages. These latter symptoms

TABLE 1. EFFECT OF DIBENAMINE ON THE ACTIVATION OF THE RABBIT ANTERIOR HYPOPHYSIS FOLLOWING COPULATION

Number of animals	Dose mg/kg	Time of injection (minutes after copulation)	Ovulated (hypophysis activated)	Failed to ovulate (stimulus blocked from reaching hypophysis)	Percentage blocked by Dibenamine
3	14-15-17	1	3	0	0
19	25-32	1	3	16*	84.2
6	25-30	3	6	0	0
3	27-29-30	5	3	0	0
2	29	12	2	0	0

* These rabbits which failed to ovulate when Dibenamine was injected immediately after mating were subsequently remated and did ovulate when either the injection of Dibenamine was delayed 3 or more minutes or Priscol was injected.

were usually most severe between two and three hours after injection, and four animals receiving doses of 27–30 mg./kg. expired at this stage, in a state of extensor rigidity. Therefore, with this speed of injection, 30 mg./kg. is above the minimal lethal dose of Dibenamine for this species. The toxic effect of this dosage is also indicated by the fact that the animals regularly lost weight during the next few days. Since none of five females surviving 30 mg./kg. would mate during the subsequent three days, post-copulatory injection of the drug was necessary.

As may be seen in Table 1, the injection of doses of 25–32 mg./kg. of Dibenamine was begun in 19 animals within a minute after the end of copulation. Of these animals, three ovulated and the remaining sixteen failed to do so. The injection of this dosage was uniformly ineffective in preventing ovulation when it was delayed for a period of

12 minutes (two animals), 5 minutes (three animals), or even for a period of 3 minutes (six animals).

Following the injection of 10–15 mg./kg. of Priscol, only a few females would mate. The three which did so (table 2) ovulated. Even with this dosage there was sometimes increased intestinal motility, as indicated by defecation, a general muscular hypotonia and wheezing respiration occasionally followed. After doses of 40–50 mg./kg. more severe symptoms were added to the above: involuntary twichings of skeletal muscles appeared, there was an extreme reduction of postural tone, and respiratory difficulty became marked. Those animals receiving 50 mg./kg. often slumped down before the dose had been completely administered and lay quite relaxed except for involuntary muscle twitches, including occasional kicks with the hind legs. They usually gasped for air for about two minutes; then they either recovered rapidly, pulling their legs beneath them and assuming their characteristic prone posture, or they expired. Three animals receiving this dosage died within 2½ minutes after injection in spite of attempts to revive them with artificial respiration. Apparently 50 mg./kg. of Priscol approaches the lethal dose (LD_{50}). However, even this amount of Priscol failed to constrict the pupil.

Of the ten animals surviving doses of 40–52 mg/kg., nine of them ovulated in spite of the Priscol (Table 2). The animal which failed to ovulate had received 40 mg./kg.; she had small ovaries completely covered with adhesions, and containing no follicles of appreciable size except for a few old hemorrhagic ones. Although she accepted the male, the appearance of the ovaries suggested that ovulation could not be expected in this rabbit.

TABLE 2. FAILURE OF PRISCOL TO BLOCK ACTIVATION OF THE RABBIT ANTERIOR HYPOPHYSIS FOLLOWING COPULATION

Number of animals	Dose mg/kg	Time of injection (minutes after copulation)	Ovulated	Failed to ovulate	Percentage blocked by Priscol
3	10-15-15	(Before copulation)	3	0	0
10	40-52*	1	9	1†	10

* Seven had 50 mg. or more per kg.
† See text, page 5.

The Priscol series, and the animals receiving Dibenamine after 3–12 minutes, offer an apparently adequate control to the positive dibenamine blocking experiments. However, in regard to failure of ovulation being due only to the general physical condition of the animal following the use of these drugs, we undertook also to determine if the deep anesthesia induced by nembutal would be effective in blocking ovulation. Each of three animals received 27 mg./kg. of

nembutal (0.4 ml. Abbot Veterinary Nembutal/kg.) in an ear vein within one minute after copulation. All three animals ovulated nevertheless.

DISCUSSION

That the animals used were in estrus and capable of ovulating is indicated both by their willingness to accept the male and by the fact that ovulation invariably followed copulation when lower or delayed doses of Dibenamine were administered or, with the single exception noted above, when Priscol or nembutal were given. In viewing these observations, the preponderant percentage of cases in which the administration of higher doess of dibenamine within a minute after coitus was followed by failure of the animal to ovulate cannot be attributed to chance. The failure of these animals to ovulate seems indubitably the result of the Dibenamine injection.

The adrenolytic and other pharmacological properties of Dibenamine have been investigated by Nickerson and Goodman (op. cit.), who report that it has a high specificity of action and that the adrenergic block which it produces is unusually complete and prolonged. Its toxic effects are reported to be primarily local tissue damage and central excitation. In the present experiments, the sympatholytic effect was obvious in the pronounced pupillary constriction. Since, however, it is possible that the blocking of ovulation produced by Dibenamine in these experiments might be due, at least in part, to the deleterious action of large doses administered quickly, this possibility must be examined.

Although the excitation and prostration induced by Dibenamine varied somewhat in detail from that produced by Priscol, it was not apparently more severe. Indeed, it was our impression that of the two drugs Priscol, in the dosages given, exerted the more profound effect upon the activity of the animal, certainly in the first few minutes following the injection. Moreover, only a very slight delay in giving the Dibenamine was sufficient to allow ovulation to proceed apparently normally, in spite of the fact that the release of luteinizing hormone from the hypophysis requires approximately an hour to be effected, and that reaction of the ovary to this hormone requires some ten to eighteen hours or more to reach its culmination. As is indicated also by the few experiments with nembutal, there seems to be no correlation between the degree of prostration or other toxic symptoms produced, and the occurrence or prevention of ovulation. It seems proper to conclude, therefore, that the action of Dibenamine in preventing ovulation from following coitus in the rabbit is due to its specific adrenolytic properties, and not to a general effect upon the animal as a whole.

Since our previous experiments indicated that ovulation could be artificially induced in the rabbit by the intrahypophyseal instillation of adrenaline, and the present ones apparently demonstrate that the

ovulation normally following coitus may be prevented by the adrenolytic action of Dibenamine, the evidence seems strong that there is a humoral link involved in the hypothalamico-hypophyseal ovulatory mechanism of the rabbit, and that this humoral link is of an adrenergic nature.

Our experiments on electrical stimulation of the hypothalamus and hypophysis suggest that the link is a relatively long one, arising outside of the anterior lobe of the hypophysis. There is as yet no definite evidence as to the locus of origin of the humoral adrenergic mediator whose action we believe we have demonstrated. The adrenal medulla can apparently be excluded from consideration in this regard; in our experiments, neither intravenous nor intracarotid injections of adrenaline were effective in producing ovulation and there is a considerable body of evidence to indicate that ovulation cannot depend upon the output of adrenaline from the adrenal. According to Nickerson and Goodman (op. cit.) Dibenamine probably acts directly upon the effector cells, and, therefore, the present experiments contribute no information upon this point. Green (1946) has pointed out that the portal system of veins of the hypophyseal stalk is admirably adapted for serving in the transmission of a neurohumoral agent from median eminence to the anterior lobe of the hypophysis. This is certainly the pathway by which one would expect specific humoral agents destined for the hypophysis to reach the gland; perhaps therefore the source of the adrenergic substance should be sought in the median eminence, rather than in the hypothalamus itself.[1] Green has also (1947) described nerve endings, of presumable hypothalamico-hypophyseal tract origin, in intimate association with the capillary loops of the median eminence, and imbedded in the sheaths of the portal veins. Whether these endings are cholinergic or adrenergic is not known, but Green has suggested that some of them may be secretory. If the nerve endings actually liberate an adrenergic substance into the portal circulation, they may be the site of the formation of an adrenergic agent arising outside of the anterior lobe which our theory postulates.

While a humoral control over the hypophysis by way of the portal circulation has been frequently suggested as a possibility, our experiments with the instillation of adrenaline into the hypophysis, and the present ones concerning the blocking of ovulation with Dibenamine, constitute, so far as we know, the first definite evidence as to the existence and nature of this control in the ovulatory mechanism of the rabbit. Eskin (1944) had previously concluded that adrenaline inhibited the secretion of luteinizing hormone in rat and rabbit. In so far as the rabbit is concerned, at least, his experiments and their interpretation are open to serious question. The dose of adrenaline which

[1] Most or all of the median eminence is regarded by Weaver and Bucy (1940) and Rioch, Wislocki and O'leary (1940) as being actually a part of the posterior lobe, rather than of the hypothalamus proper.

he administered was close to the lethal dose for a rabbit of average size; it is somewhat surprising that even four out of eleven animals receiving this dose would mate within the following 15 minutes, and the lack of ovulation in these four (although three were said to have hemorrhagic follicles, probably an indication of some hypophyseal stimulation) can hardly in this case be attributed to specific inhibitory action of adrenaline upon the hypophysis.

In regard to the non-effect of Priscol in blocking ovulation, it should be pointed out that this drug possesses many different pharmacological actions, including both sympathomimetic and sympatholytic qualities (Ahlquist et al., 1947). As judged by our results with Dibenamine, the action of Priscol upon the anterior pituitary gland is not predominantly adrenolytic. The one case in which ovulation failed to occur following the administration of Priscol is presumably due to the condition of the ovaries rather than to the action of this drug.

While it has been impossible to establish physiologically exact times within our experiments, they give evidence as to the approximate time during which stimulation of the hypophysis is necessary in order to produce a liberation of luteinizing hormone adequate for the production of ovulation. While Fee and Parkes (op. cit.), and others have shown that the liberation of the luteinizing hormone from the anterior hypophysis requires approximately an hour, our experiments indicate that the stimulation necessary to produce this liberation is of the order of no more than one to three minutes. Dibenamine in adequate doses given within a minute after the end of coitus rather effectively blocked ovulation, while it was ineffective when its administration was delayed for 3 minutes or more.

The results presented here, therefore, reenforce our belief that an adrenergic humoral agent is normally concerned in the liberation of luteinizing hormone from the hypophysis of the rabbit following coitus, and indicate that this humoral agent produces its full effect upon the hypophysis within a very few minutes. The delay in the release of luteinizing hormone is apparently due to the necessary adjustments within the glandular cells, and not to the necessity of repeated or very prolonged stimulation of these cells.

Certain other events in the ovulatory mechanism may be given time values. Claesson and Hillarp (1947) have found that within 3 to 5 hours after mating most of the sterol granules are mobilized from the interstitial gland of the rabbit ovary. Markee (1933) reported that enough estrogen had reached the endometrium to induce vasodilatation in intraocular transplants within about 7 hours of mating. The events in the ovulatory mechanism, in the rabbit, proceed at about the following speed: stimulation of the hypophyseal cells requires about one to three minutes; an adequate amount of L. H. is discharged in about an hour; the cholesterol like sterols are mobilized from the interstitial gland of the ovary within 3 to 5 hours; estrogen sufficient to induce dilatation of endometrial vessels is secreted in

about 7 hours; and, the follicles rupture about 10 to 14 hours after mating.

SUMMARY

In previous experiments, we found that ovulation can be induced in the rabbit by the instillation of adrenaline into the hypophysis, and advanced the theory that the ovulatory mechanism includes an adrenergic humoral link between hypothalamus and anterior hypophysis. As a test of this hypothesis, we have, in the present experiments, tried to determine whether the ovulation normally following coitus in the rabbit can be blocked by some of the available sympatholytic and adrenolytic drugs.

Twenty-five to thirty-two mg./kg. of Dibenamine, given intravenously within 1 minute after the end of copulation, prevented ovulation in sixteen of nineteen animals. The same doses of Dibenamine were ineffective if injection was delayed for three minutes or more following coitus. These experiments support the hypothesis that ovulation in the rabbit is dependent upon a humoral pathway of an adrenergic nature, and indicate that the necessary stimulation of the hypophysis is completed within little more than one minute following copulation.

Priscol was found to be ineffective in preventing ovulation following coitus.

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THE SPONTANEOUS FORMATION OF INTRACELLULAR COLLOID DROPLETS IN SURVIVING CHICK THYROID TISSUE[1]

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RECENTLY, Junquiera (1947) reported the formation of large numbers of intracellular colloid droplets in rat thyroid tissue exposed in vitro to thyrotrophic hormone. None formed in control thyroid tissue. Since earlier studies had indicated that these droplets formed after the parenteral administration of pituitary gland preparations to a variety of species (Grant, 1930, 1931; Severinghaus, 1933; Okkels, 1934; Aleschin, 1935, 1939; De Robertis, 1941 a, 1942), the in vitro experiments were viewed by Junquiera as evidence of a direct action of thyrotrophic hormone on thyroid epithelium.

Since droplet formation in chick thyroids had earlier been used as a basis for thyrotrophic hormone assay in intact chicks (Dvoskin, 1947), an attempt was made to determine if it would be feasible to assay thyrotrophic hormine, in vitro, by exposing chick thyroid tissue to thyrotrophic hormone. To our surprise it was observed that control chick thyroid glands formed large numbers of intracellular colloid droplets in vitro in the absence of added thyrotrophic hormone. Similar observations had been made earlier in other species by Langendorff (1889), Lubcke (1902), and Champy (1915), who described the infiltration of the follicular epithelium by the colloid as it was resorbed from the follicular lumen. Demuth (1932) noted the formation of fuchsinophilic bodies in thyroid epithelium of chick embryos in vitro.

Because the in vitro behavior of surviving thyroid tissue was similar to that of the thyroids of intact chicks treated with thyrotrophic hormone, as regards formation of intracellular colloid droplets, it was decided to investigate this response. An understanding of the conditions under which the in vitro reaction occurs might provide some clues to the mechanism by which thyrotrophic hormone evokes formation of intracellular colloid droplets.

MATERIALS AND METHODS

One to five day-old White Leghorn cockerels[3] were used. They were

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[3] Obtained from Hall Brothers Hatchery, Inc., Wallingford, Conn.

housed in a brooder kept at about 30° C. Groups of three to six chicks were used for each experiment. The chicks were killed by decapitation, and the thyroid glands were rapidly dissected free. In most experiments one thyroid gland was immediately fixed in Carnoy's fluid,[4] and served as a control. The other gland was then used in the experiment, and at the end, it was also fixed in Carnoy's for one hour. The thyroids were then rapidly embedded in paraffin and sectioned through the center at 4 μ. Sections were stained with the modified Heidenhain-Azan (Dvoskin, 1947).

The in vitro experiments were conducted so as to study the influence of a variety of factors on the formation of intracellular colloid droplets. After removal from the chicks, the thyroids were placed in individual 5 cc. glass stoppered vials containing 0.5 cc. of either chick, human or rat serum or Tyrode's solution. Since these media were similar in their action, and since Tyrode's solution was most readily available, most of the following experiments were conducted with Tyrode's as the incubating medium. Earlier experiments, testing the influence of time on the reaction, had been conducted for 1, 2, 4, or 8 hours. Two hours had been found adequate to allow formation of large numbers of droplets, and this time was used in all subsequent experiments. Most experiments were conducted at 38° C. Other experiments were done at 5° C., 40° C., or 43° C.[5]

The effect of the supernatant gas on the reaction was tested by incubating groups of thyroids at 38°C. under either air, 100% oxygen, 100% nitrogen, 100% carbon dioxide, 10% carbon dioxide and 90% oxygen, alveolar air, or illuminating gas. The thyroids were agitated at 5 minute intervals in their vials for 2 hours. Since the nature of the supernatant gas failed to influence the response, the following experiments were conducted under air.

One group of experiments was designed to test the effect of the saline concentration of the medium on the reaction. Thus, groups of thyroid were incubated for 2 hours at 38°C. in either distilled water, Tyrode's solution, Tyrode's solution diluted 50% with distilled water, Tyrode's solution concentrated by 50%, or in a solution of 1.8% NaCl in distilled water.

In another group of experiments, the effect of the pH of the medium was investigated. Tyrode's solution, which had a pH of about 7.4 at the start of the experiment, was used.[6] This medium was made strongly alkaline to pH 12, and strongly acid to pH 2, by adding concentrated HCl and NaOH. In other experiments the pH was varied moderately by changing the concentration of carbon dioxide in the supernatant gas, and these experiments are identical with those reported above in which the effect of the supernatant gas was studied.

In other experiments, the effect of a variety of chemical agents, known to influence either thyroid function or enzyme activity, was studied. The following agents were added to Tyrode's solution in the following concentrations: NaI, 0.01 M; NaI, 0.007 M plus I_2, 0.003 M; d, 1-thyroxine, 0.00014 M; thiouracil, 0.01 M; thyrotrophic hormone,[7] 0.066 chick wt. u. in 0.5

[4] This fixative was earlier shown to be able to preserve large numbers of intracellular colloid droplets in chick thyroids after thyrotrophic hormone administration (Dvoskin, 1947).

[5] The cockerels' rectal temperature was about 40°C.

[6] Solutions used as incubating media became progressively more alkaline during incubation. The pH changed from one to two units during a two hour incubation period.

[7] A flavianic acid precipitate of hog pituitary glands prepared by Dr. George Smelser was used. This preparation was assayed on the basis of intracellular colloid droplet formation in intact chicks in an earlier paper (Dvoskin, 1947).

cc.; NaCN, 0.001 M; KSCN, 0.001 M; NaF, 0.001 M; $KMnO_4$,[8] 0.0001 M. The thyroids were incubated in 0.5 cc. of these media for 2 hours at 38°C. under air.

Because the droplets appeared first in the central follicles, it was decided to determine whether this depended on the position of the follicle in relation to the mass of thyroid tissue, or on the inherent increased activity in central thyroid follicles.[9] Chick thyroids were cut in half with a sharp razor, and incubated at 38°C. for two or eight hours under air in Tyrode's solution. After paraffin embedding, the glands were sectioned at right angles to the plane of the original section.

In order to determine whether the droplets in the cells represented colloid entering from the follicular colloid or whether it was colloid forming in the cells, a limited number of observations were made of thin sections of surviving thyroid tissue placed in a moist chamber using a microscope with a 10 × ocular and a 1.9 mm. oil immersion objective.

In one group of experiments the thyroid glands were allowed to remain in the chicks. In some, the thyroids of decapitated chicks were kept in situ at 38°C. for two hours. In others, the thyroids remained in chicks which were allowed to live for two hours after the following procedures, performed under ether anesthesia. In one group, the common carotid artery and jugular vein were ligated above and below the thyroid on the left side. In another, these vessels were severed after ligation. In another group, the vessels were severed, and the left thyroid and surrounding tissues were dissected free of all but a thin stalk of surrounding connective tissue. The block of tissue was then allowed to remain in situ. In another group, the block of tissue including the left thyroid was transplanted to the subcutaneous tissue on the opposite side of the neck. In still another group, the left thyroid gland was freed of all surrounding connective tissue and similarly transplanted. At the end of two hours, the chicks were decapitated, and the thyroids treated in a manner similar to that described above. The right thyroid was also removed at autopsy, and served as a control.

RESULTS

The type of response most commonly observed is exemplified by that of the thyroids incubated at 38°C. for two hours in Tyrode's solution under air. The formation of intracellular colloid droplets was prominent in the central follicles (Fig. 1) and did not occur in the peripheral 2 to 8 rows of follicles. The intracellular colloid droplets ranged from about a micron, or less, to several microns in diameter. The smaller droplets occurred in the apical parts of the cells. The large colloid droplets, which formed more abundantly, were frequently so large as to fill entire cells. Only an occasional strand of colloid appeared to connect the intracellular and intrafollicular colloid. The intrafollicular colloid frequently failed to fill completely the follicular lumen, and assumed a somewhat central position. In other cases the

[8] The $KMnO_4$ was used to determine whether an oxidizing agent, similar to elemental iodine in this regard, would have a similar action. No glucose was added to the Tyrode's solution when $KMnO_4$ was used, since the $KMnO_4$ was rapidly reduced in its presence. Thyroid tissue slowly reduced both I_2 and $KMnO_4$ as evidenced by the progressive decolorization of these solutions.

[9] This evidence is presented by Dempsey and Singer (1946).

intrafollicular colloid had a scalloped margin. Frequently, however, numerous droplets were present in follicles which had well preserved colloid which was closely approximated to the epithelial border. Control thyroid tissue rarely had any intracellular colloid droplets (Fig. 2). The nuclei of incubated as well as those of control thyroids appeared clear and vesicular and were not pycnotic. Occasionally, nucleoli stained like droplets of intracellular colloid.

The reaction occurring during incubation, though similar to that after thyrotrophic hormone administration, differed from it in several important respects. The cell height was not increased by the incubation.[10] Nor were non-staining vacuoles present in the cells, such as commonly seen after stimulation with thyrotrophic hormone (Dvoskin, 1947). Also, colloid droplets were much larger than those ever seen even with intense stimulation with thyrotrophic hormone.

The duration of incubation exerted a profound influence on the formation of intracellular colloid. Colloid droplets were present in the central follicles after incubation for one hour, and were more numerous after two or four hours. After incubation for eight hours, only a peripheral zone of 2 to 8 rows of intact follicles remained, and no intracellular colloid droplets formed here (Fig. 5). The centers of the glands were largely necrotic, although the shape of the follicles was preserved. The nuclei and numerous large intracellular colloid droplets remained, marking the position of the cells, but intrafollicular colloid was almost completely absent. Strangely, colloid depletion was less prominent in the very center than in the surrounding zone (Fig. 5). In the zone between the necrotic core and the intact periphery, several rows of follicles still were intact, and contained numerous intracellular colloid droplets. The necrosis of the center of the glands, observed after 8 hours' incubation, corresponds almost exactly with that described in thyroid transplants at the end of 24 hours (Holdener, 1898; Sultan, 1898; Lubke, 1902; Hesselberg, 1915; Tureen, 1927; Weil and Bernheim, 1936).

A temperature of 5°C. completely inhibited the formation of intra-

[10] Cell height increases after the administration of large dosages of thyrotrophic hormone. However, droplets may form without any increase in cell height, when low dosages of thyrotrophic hormone are administered (Dvoskin, 1947).

EXPLANATION OF PLATE 1

FIG. 1. Central follicles of left thyroid of a three-day-old chick incubated for two hours at 38°C. under air in Tyrode's solution. ×450.

FIG. 2. Central follicles of right thyroid of chick whose other thyroid is shown in Fig. 1. This gland was fixed at autopsy. ×450.

FIG. 3. Peripheral follicles of left thyroid of a two-day-old chick incubated for two hours at 38°C. under air in Tyrode's solution containing 0.066 Ck. Wt. U. of thyrotrophic hormone in 0.5 cc. ×690.

FIG. 4. Peripheral follicles of right thyroid of chick whose other thyroid is shown in Fig. 3. This gland was incubated under conditions identical to those used for the thyroid shown in Fig. 3, except that the solution containing thyrotrophic hormone had been heated to 100°C. for ten minutes prior to incubation. ×690.

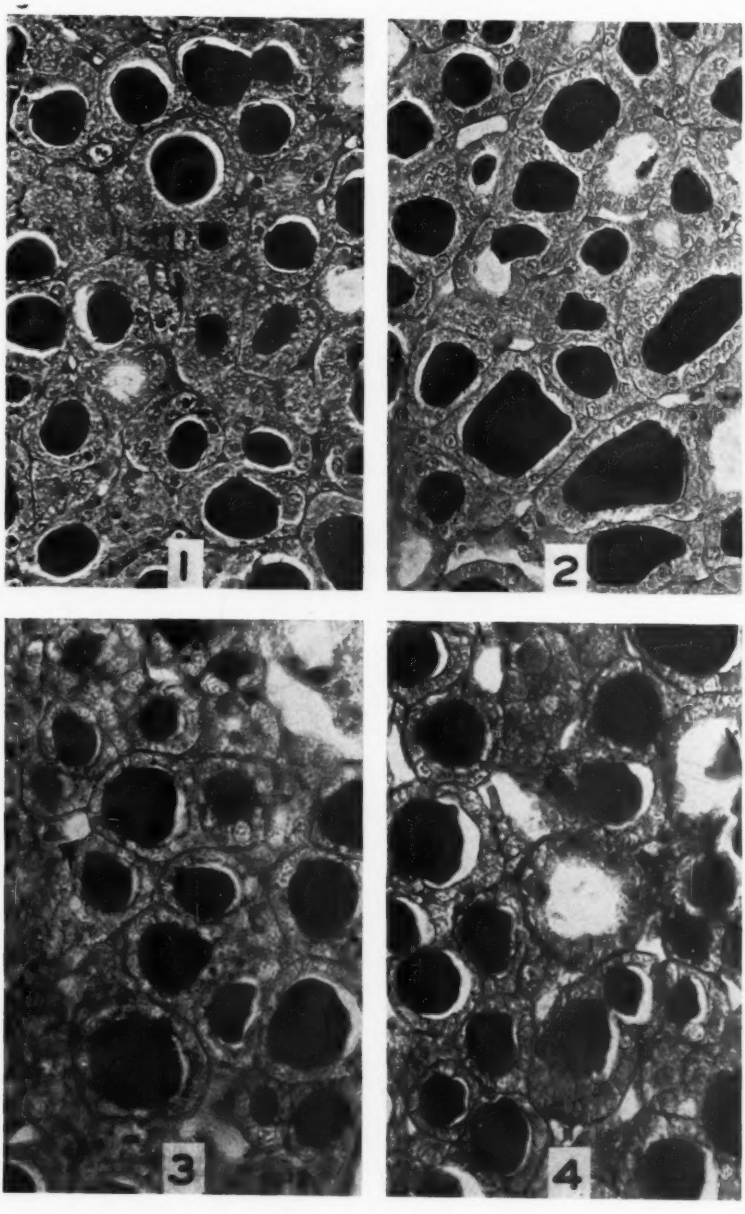

PLATE 1

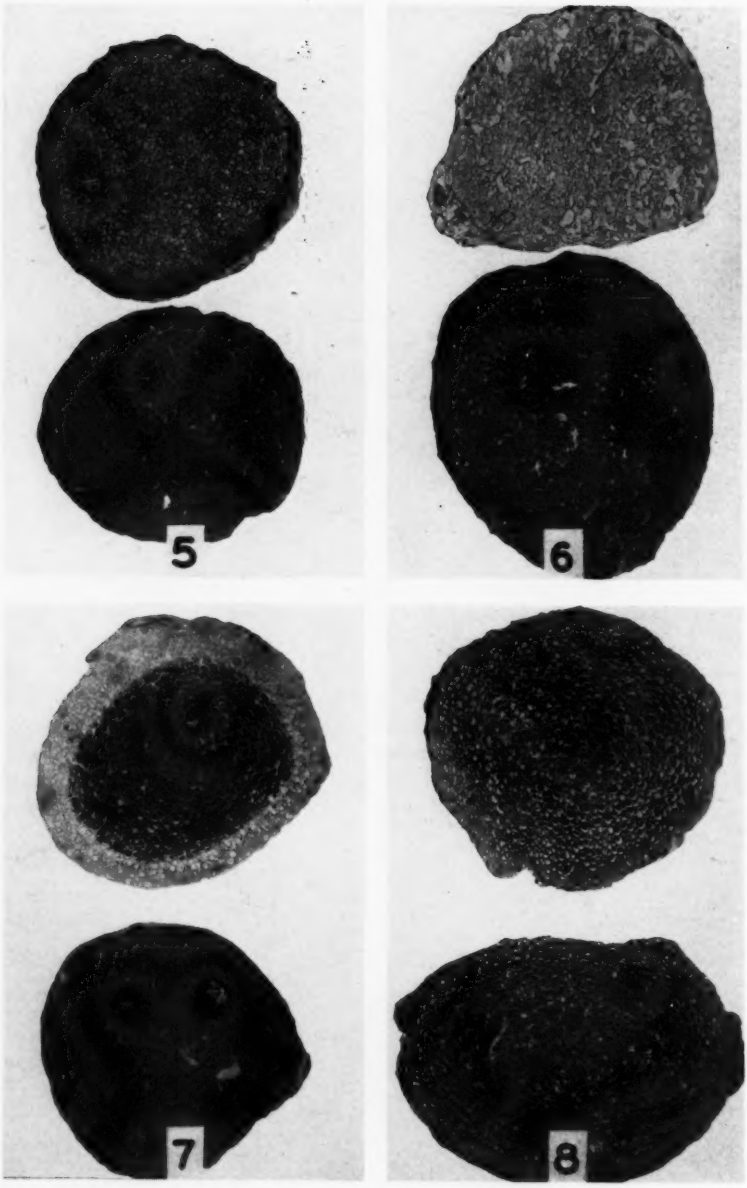

PLATE 2

cellular colloid droplets for two hours, and thyroids kept at this temperature in Tyrode's were indistinguishable from control glands fixed at autopsy. Nor was the cell height increased by incubation at low temperatures as implied by Turner and Turner (1945). Incubation at temperatures of 40°C. and 43°C. for two hours resulted in the formation of large numbers of intracellular colloid droplets in the centers of the glands, sparing the most peripheral follicles.

The nature of the incubating medium, such as the sera from different species or Tyrode's solution, did not alter the reaction, and numerous droplets formed in the centers of the glands. Nor did the nature of the supernatant gas alter the above-described response at the end of two hours. Of interest is the fact that even the complete anoxia due to incubation under 100% nitrogen or carbon dioxide or illuminating gas did not induce droplet formation in the peripheral follicles.

Hypotonic solutions as incubating media markedly altered thyroid structure. Those thyroids incubated for two hours in distilled water were completely depleted of colloid and had remaining only the ghost of the follicular connective tissue, and a network of debris filling the follicle. Intracellular colloid droplets were not present (Fig. 6). Occasionally, nuclei remained in some follicles. When Tyrode's solution diluted 50% was used as the incubating medium, the central follicles were noted to be markedly depleted of colloid, and occasional intracellular droplets formed. The colloid did not appear depleted in the peripheral follicles. Surprisingly, cell height was not increased, as might be anticipated with hypotonic media (Plagge, 1943; Williams, 1944).

The hypertonic media resulted in an increased affinity of the thyroids for the aniline blue stain, and sections were much darker than control tissue mounted and stained on the same slide. No depletion of colloid was noted. Many large intracellular colloid droplets formed when Tyrode's solution, concentrated by 50% was used. These large droplets occasionally formed in follicles at the periphery of the gland. Droplets rarely formed in the gland when 1.8% NaCl was

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EXPLANATION OF PLATE 2

Sections of thyroids of two-day to four-day old chicks. Each figure shows both thyroids from a single chick. The lower thyroid in each figure was fixed at autopsy and served as a control. The upper thyroid in each figure was incubated at 38°C. under air in the following solutions. ×32.

FIG. 5. Incubated in Tyrode's solution for eight hours.

FIG. 6. Incubated in distilled water for two hours.

FIG. 7. Incubated in Tyrode's solution containing 0.007 M NaI and 0.003 M I_2 for two hours.

FIG. 8. Incubated in Tyrode's solution containing 0.001 M NaCN for two hours.

The dark staining portions of the thyroids are those containing intrafollicular colloid. The light staining portions are depleted of colloid. Normal control thyroids stain darkly because of their high content of colloid.

used. Cell height was much reduced by incubation in hypertonic media. Indeed, the inhibition of droplet formation and the lowering of cell height in vitro by strong concentrations of sodium iodide reported by Junquiera (1947) may well be due to the hypertonicity of the incubating medium rather than to a specific inhibitory effect by iodide.

The extremes of pH prevented the formation of intracellular colloid droplets in the thyroid, probably by rapidly killing the tissues. The tissues stained darkly. No colloid depletion occurred. A pH of 5.2 6.2, or 7.2, produced in Tyrode's using 100% carbon dioxide, 10% carbon dioxide plus 90% oxygen or alveolar air as the supernatant gases, failed to alter the response usually observed in Tyrode's solution under air or oxygen.

None of the group of chemical agents added to Tyrode's solution inhibited the formation of intracellular colloid droplets, except for elemental iodine. With this latter agent, the peripheral half of the gland had follicles which were completely depleted of colloid. The glands presented an unusual appearance in that a sharp demarcation was noted between the outer light staining and inner dark staining halves of the gland (Fig. 7). In the central follicles, only a few colloid droplets had formed. Iodide alone did not have this effect.

NaI, NaCN, KSCN, NaF, $KMnO_4$, thiouracil and thyroxine failed to inhibit the formation of intracellular colloid. Contrary to what usually occurred with other chemicals, incubation with NaCN resulted in colloid depletion in the peripheral follicles, whose shape was distinguishable by the remaining circular rows of nuclei and colloid droplets (Fig. 8). With the other agents, colloid depletion occurred in the central rather than in the peripheral follicles. Droplet formation occurred in the central follicles, but not in the peripheral follicles.

The experiments with thyrotrophic hormone were conducted so that control thyroids were incubated in a medium containing the identical amount of thyrotrophic hormone, except that the control solution had been heated to 100°C. for 10 minutes prior to incubation.[11] Other control thyroids were incubated in Tyrode's solution alone. In both control and experimental groups, many large intracellular colloid droplets formed in the central follicles. However, only the thyroids incubated in the unheated thyrotrophic hormone solution had numerous small apical droplets in the peripheral follicles (Figs. 3 and 4). A zone of follicles which had no droplets within the cells separated these two zones.

In the experiments with halved thyroid glands, the most striking

[11] Heating destroys thyrotrophic hormone (Rowlands and Parkes, 1934; Anderson and Collip, 1934; Collip, 1935; Stimmel, McCullagh and Picha, 1936; Rawson, Sterne and Aub, 1941). Other evidence denies this (Krichesky, 1934; Rawson, Sterne and Aub, 1941).

droplet formation occurred at the end of two hours in the central portions of each half gland, sparing the entire peripheral zone, even though one portion of that zone had earlier been in the center of the intact thyroid. With incubation for 8 hours, only the peripheral follicles remained intact, and there was necrosis of the follicles in the center of each half thyroid, and not in the zone that had earlier been in the center of the gland.

The experiments in which in vitro observation of chick thyroid tissue was attempted were unsatisfactory since no intracellular colloid droplets were seen with the ordinary microscope.

When the thyroids were allowed to remain intact in a decapitated chick for two hours at 38°C., a small but definite number of intracellular colloid droplets formed. In the living chicks, no droplets formed in the thyroids which were rendered partially ischemic by ligation, or by ligation and severance of the common carotid and internal jugular veins. Only when the thyroids were freed of most or all connections with surrounding structures were large numbers of droplets formed. These also were abundant in the thyroids transplanted to the opposite side of the neck. None appeared in the control undisturbed gland on the right side of the neck. Many more droplets formed in the thyroids rendered completely ischemic by homotransplantation than in those allowed to remain in situ in the decapitated chicks. Possibly the trauma incident to dissecting the thyroids was a factor stimulating droplet formation. Williams (1944) noted droplet formation in living thyroid cells after a micro-needle was introduced into the cell base.

DISCUSSION

From the above results, it appears that intracellular colloid droplet formation occurred chiefly and primarily in the ischemic central mass of thyroid tissue, both in the in vitro, and in the in vivo experiments. At the end of 8 hours of incubation the process advanced to frank necrosis in the centers of the glands.

Droplet formation was stopped or largely inhibited by low temperature. Also, strong reagents, such as strong alkali or acids or strongly hypertonic saline or elemental iodine, which probably killed the tissue, prevented droplet formation. None of the other chemical agents added to the media inhibited this reaction. Possibly these failed to penetrate the entire gland, although such media as hypotonic or hypertonic Tyrode's or strongly acid or basic Tyrode's affected the glands throughout. Thyrotrophic hormone induced formation of small droplets in the peripheral row of follicles, a zone usually having none, although occasionally large droplets formed in this zone in thyroids incubated in hypertonic Tyrode's or in $KMnO_4$.

The rapid post mortem autolysis of thyroid tissue has been the subject of numerous investigations (Kolliker, 1854; Verson, 1872;

Langendorff, 1889; Lubcke, 1902; Champy, 1915; Marine, 1915; Guillebeau, 1917; Ferrero, 1925; Gloor, 1926; Murray, 1927; Thomas, 1934; Ewers, 1936; Bargmann, 1939). Most of the above observers commented on the depletion of colloid which occurs post mortem. Some observed failure of new colloid formation (Demuth, 1932). Others observed colloid droplet formation within desquamated epithelial cells (Wail, 1923; Abrikosoff, 1924; Trautman and Fiebiger, 1931; Aleschin, 1935). Ebeling (1925) found that intrafollicular colloid persisted or formed in small pieces of explanted chick thyroid for as long as four months. Only Langendorff (1889), Lubcke (1902) and Champy, (1915) speak of colloid infiltrating the cells. Champy observed small droplets in thyroid cells in tissue culture, which stained somewhat like colloid. He viewed this as evidence of a transcellular passage of intrafollicular colloid. Although this view is becoming accepted (Severinghaus, 1933; Ponse, 1938; De Robertis, 1941 a), in vitro or direct observation of transplants of thyroid tissue still provide evidence that intracellular colloid may represent material forming in cells to be discharged into the follicular lumen (Verson, 1872; Williams, 1937, 1944).

Two factors may be suggested to explain the formation of intracellular colloid in surviving thyroid tissue. First, the cells may have adherent thyrotrophic hormone which becomes activated under the asphyxial and possibly reducing conditions in the center of the gland. The enhanced potency of thyrotrophic hormone after the addition of reducing agents (Albert, Rawson, Merrill, Lennon and Riddell, 1947) is suggestive. However, no droplets formed in the peripheral thyroid follicles when the glands were incubated in the reducing environment of thiouracil media. Secondly, proteolytic enzymes, contained in thyroid tissue (De Robertis, 1941 b; Dziemian, 1943; De Robertis and Nowinski 1946 a), may be related to droplet formation. The proteolytic enzyme content of thyroid tissue is increased after thyrotrophic hormone administration (De Robertis, 1941; Dziemian, 1943), and in hyperthyroidism (De Robertis and Nowinski, 1946 a). Intracellular colloid droplets also appear in thyroid cells under these conditions (Aleschin, 1935, 1939; De Robertis, 1941 a; 1942; De Robertis and Del Conte, 1944; Dvoskin, 1947). Proteolytic enzymes of the cathepsin type are known to be of importance in autolyzing tissue, and are most effective in reducing environments (Sumner and Somers, 1947). Intracellular colloid droplet formation and colloid depletion were found to parallel autolysis in these experiments. Trauma of the tissues, which is known to increase autolysis, is also suggested as a factor stimulating colloid droplet formation. These reactions may have a common basis in the post-mortem activation of proteolytic enzymes.

Rapid autolysis occurs in thyroid tissue of mammals as well as chicks. Entire rat thyroids suffer colloid depletion in central follicles

after incubation for 20 to 40 minutes at 37°C. in Tyrode's. At the end of 90 minutes, only a peripheral zone of 2 or 3 rows of follicles remains intact. Large intracellular colloid droplets are also formed.

The experiments, in which elemental iodine was shown to cause the depletion of colloid from peripheral follicles, which ordinarily maintain their intrafollicular colloid content, contradict the thesis of De Robertis and Nowinski (1946 b) that elemental iodine inhibits colloid release by interfering with the activity of the proteolytic enzymes of thyroid tissue. Also, the absence of intracellular colloid droplets in these follicles contradicts our hypothesis that proteolytic enzyme activity may be related to colloid release and formation of intracellular droplets. However, other factors, such as lethal injury to follicular epithelium may account for colloid release, and for the colloid depletion of the follicles of thyroids incubated in distilled water or Tyrode's containing NaCN, as well as of thyroids incubated in Tyrode's containing elemental iodine.

The rapid autolysis of thyroid tissue has several implications for in vitro work. Marine (1915) noted a loss of iodine from surviving thyroid tissue, after it had been perfused for some time. Iodine loss also occurs in thyroids after vaso-ligation (Watts, 1915). Since thyroidal material might also be liberated from thyroid slices during its necrosis, and since thyroxine has an inhibitory effect on some of the responses due to thyrotrophic hormone (Cortell and Rawson, 1944), this might partly explain the inactivation of thyrotrophic hormone known to follow in vitro incubation of the hormone and thyroid tissue[12] (Seidlin, 1940; Rawson, Sterne and Aub, 1941, 1942). Also, with in vitro studies attempting to establish quantitative estimates of thyroid activity, based on inactivation of thyrotrophic hormone, or on formation of thyroidal materials, or on oxygen consumption, one should bear in mind that only a fraction of the thyroid tissue remains alive when slices as thin as 0.5 mm. are used.[13] Surface area rather than weight would be the best guide in estimating the amount of tissue participating in the reaction. Although an attempt to assay thyrotrophic hormone in vitro by a study of the intracellular droplets may succeed with minute fragments, most certainly the abundant formation of droplets in vitro in slices as thin as 0.5 mm., when no thyrotrophic hormone is added, should cast suspicion on any but the most well controlled studies.

The spontaneous formation of intracellular colloid droplets must

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[12] This possibility fails to explain why lymphoid tissue and thymus, which wouldn't be expected to contain thyroidal material, also partially inactivate thyrotrophic hormone (Rawson, Sterne, Aub, 1941). Also, hyperthyroid tissue, which contains but little colloid and little organic iodine (Gutman, Benedict, Baxter and Palmer, 1932) has strong inactivating properties (Rawson, Graham and Ridell, 1943).

[13] The entire chick thyroids used in these experiments were about 1 mm. in diameter. The halves were 0.5 mm. in diameter, and necrosis occurred in the centers of even these thin slices.

also be considered in all studies of these structures in thyroid tissue which is not immediately fixed or refrigerated promptly after removal at operation.

SUMMARY

The intact thyroid glands of two to five day-old cockerels were incubated in vitro in a variety of media, for one, two, four or eight hours, at 5°C., 38°C., 40°C., or 43°C., under an atmosphere of either nitrogen, oxygen, carbon dioxide or a mixture of these three gases, or under illuminating gas. Various chemical agents were added to the incubating medium including I_2, NaI, thyroxine, thiouracil, thyrotrophic hormone, NaCN, NaF, KSCN, or $KMnO_4$ in 0.01 to 0.0001 M concentrations. Some experiments, designed to render the thyroids progressively ischemic, were done on intact thyroids in living chicks. Control glands were studied in all cases. The thyroids were fixed in Carnoy's fluid, and sections were stained to demonstrate the intracellular colloid droplets and the intrafollicular colloid.

Numerous large droplets formed under most conditions in the absence of added thyrotrophic hormone. Droplet formation occurred almost entirely in the center of the gland, rarely in the peripheral follicles, and was maximal after 2 to 4 hours. Colloid loss occurred in the central follicles, while colloid was preserved in peripheral follicles. Droplet formation and colloid depletion were pronounced for as long as 8 hours of incubation, but the core of the thyroids became partially necrotic. The reactions in the center of the gland depended on the inner position of the follicles rather than on any inherent increased activity of the center of the gland.

Droplet formation and colloid depletion were completely prevented by incubation at 5°C., or by incubation at 38°C. in strongly acid or alkaline or strongly hypotonic or hypertonic solutions. Elemental iodine (I_2) inhibited droplet formation. All other chemical agents, added to the medium or to the supernatant gas, failed to halt the reaction, although possibly they did not diffuse into the centers of the glands. The addition of NaCN and I_2 to the incubating media resulted in depletion of colloid in the peripheral follicles.

Thyrotrophic hormone stimulated the formation of small intracellular colloid droplets in the peripheral follicles of the thyroid, a zone always free of them in the incubated thyroids. Occasionally, large colloid droplets formed in peripheral follicles incubated in hypertonic Tyrode's solution or in Tyrode's solution containing $KMnO_4$.

In living chicks, intracellular colloid droplets formed only in completely ischemic thyroids. No droplets formed in glands made partially ischemic for 2 hours. Thyroid glands, not disturbed by dissection, and allowed to remain in situ in decapitated chicks, contained some

intracellular colloid droplets, but they definitely were fewer than in other groups.

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STABILIZATION OF THE ALCOHOLIC POTASSIUM HYDROXIDE IN COLORIMETRIC 17-KETOSTEROID DETERMINATIONS[1]

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THE 2.5 normal potassium hydroxide in absolute ethanol employed in the Callow modification of the Zimmermann reaction (Callow et al., 1938), cannot be used for more than a few days after preparation. A marked increase in the color developed in reagent blank mixtures indicates deterioration even before the solution turns yellow. Various observations suggested that these changes are primarily oxidative. It therefore appeared probable that a suitable reducing agent would stabilize the solution. Experimental findings described below reveal that incorporation of ascorbic acid in the reagent, and maintenance of an atmosphere of nitrogen, yield preparations which remain stable for at least 3 months.

Preparation of 2.5 N Alcoholic Potassium Hydroxide Protected with Ascorbic Acid and Nitrogen.—One hundred ml. of absolute alcohol are measured into a glass stoppered Pyrex bottle chilled in crushed ice. Two lots of 15 mg. each of ascorbic acid are weighed. The first lot is ground up with small successive portions of absolute alcohol and added to the chilled alcohol. Since ascorbic acid is sparingly soluble in ethanol it is added as a fine suspension, and this must be done before dissolution of the solid potassium hydroxide and again after filtering the resultant cloudy solution. Twenty-five to 30 grams of finely powdered potassium hydroxide pellets are next added. A stream of nitrogen is gently bubbled through the mixture for about 5 seconds, the stopper replaced under nitrogen, and the bottle shaken vigorously for about 5 minutes. It is necessary to shake until a concentration of at least 2.6 N is reached. After sampling to test the normality the air space is again flushed with nitrogen if further shaking is required. The solution is then filtered through a fine porosity Buchner type Pyrex glass fritted filter into a 250 ml. Pyrex suction flask packed in crushed ice. A gentle stream of nitrogen is played over the surface of the filtering fluid from a conical funnel. The second lot of 15 mg. of ascorbic acid is ground with absolute alcohol and added to the filtered solution. The final concentration of the cold alkali is then adjusted to 2.5 N within 1%. A slight turbidity from the ascorbic acid may be disregarded. The reagent is stored in the cold under nitrogen. Thereafter the air

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space is always flushed with nitrogen after the bottle has been opened.

Properties of the Protected Alcoholic Alkali.—The stability of each preparation was tested by measuring the absorption of reagent blank mixtures in comparison with 70% alcohol alone. Table I illustrates the constancy of blank absorption values at various time intervals after preparation of the reagent. Eighty-seven days later the absorption had increased almost negligibly from an initial value equivalent to about 11 μg. dehydroisoandrosterone to a final value of approximately 13 μg.

TABLE I. ALCOHOLIC ALKALI PROTECTED WITH ASCORBIC ACID AND NITROGEN: EFFECT OF AGE OF THE REAGENT ON THE BLANK ABSORPTION AND ON THE OBSERVED 17-KETOSTEROID CONTENT OF VARIOUS URINE FRACTIONS

			Ketonic	High ratio	Low ratio				♀ Addison's		Non ketonic	
					Alone	+D			Alone	+D	Alone	+D
Average 520/420 ratio			1.96	1.92	0.64	0.95			0.57	0.98	0.65	1.10
Days	Blank L	K_D	μg. 17-Ketosteroid Equivalent in Sample				Blank L	K_D	μg. 17-Ketosteroid Equivalent in Sample			
1	0.094	8.8	27.5	25.9	24.3	42.2	0.097	8.7	—	—	17.4	36.2
2	0.093	8.8	29.6	25.4	24.6	41.4	0.097	8.8	16.5	34.4	16.7	35.6
4	0.100	8.9	28.9	25.4	24.9	41.3	0.098	9.1	15.3	32.4	17.2	33.8
7	0.090	8.9	29.0	25.4	25.0	41.0	0.096	9.1	15.2	32.8	16.7	35.0
11	0.089	8.9	29.2	25.4	24.8	41.3	0.102	9.0	16.2	33.8	17.1	35.5
16	0.102	8.9	26.5	24.8	24.7	40.6	0.097	8.9	16.0	33.2	17.2	35.2
23	0.094	9.0	27.0	25.3	25.6	41.3	0.097	9.0	16.1	32.0	14.0	36.2
30	0.101	9.2	28.4	24.9	26.2	40.6	0.105	9.0	15.6	33.0	18.1	36.4
35	0.102	8.9	28.9	26.3	25.0	42.5	0.114	8.6	15.7	33.9	18.0	34.6
42	0.108	9.2	29.0	24.4	25.1	41.0	0.112	9.1	14.6	33.0	16.7	33.8
63	0.116	9.1	28.1	25.6	24.6	42.0	0.125	9.2	14.9	32.4	15.4	34.6
77	0.105	8.9	28.6	26.0	26.8	42.5	0.102	9.1	—	34.5	17.2	35.0
87	0.119	9.3	27.2	24.8	25.9	41.0	0.119	9.0	15.5	32.0	16.7	36.8

The high ratio, low ratio, and Addison's fractions are whole neutral fractions of urine extract.
The ketonic and non ketonic fractions were prepared from whole neutral fractions with Girard's reagent T.
D is dehydroisoandrosterone. 20 μg. D was added to the same volume of extract as was run alone. Thus non ketonic +D is .04 ml. non ketonic plus 20 μg. D.
K_D is the extinction of 1 mg. of dehydrosioandrosterone. It is calculated by multiplying the L value for 30 μg. D by 33.3.
L is 2 −log galvanometer reading on the Evelyn photoelectric colorimeter using #520 filter.
Blank L is that obtained when reagent blank tubes were read against a "blank" containing only 70% alcohol.
The μg 17-ketosteroid equivalent in each sample was calculated using the K_D obtained at the same time.

It was necessary to prove that the ascorbic acid does not interfere with the Zimmermann reaction, and also that during the period when the blank absorption remains virtually unchanged, no other deterioration occurs which would alter observed 17-ketosteroid values. The experiments described below show that the reaction is not altered by ascorbic acid or nitrogen. Chromogenic capacities of 5 crystalline 17-ketosteroids as well as those of 17-ketosteroids contained in various urine extracts were not significantly altered by the presence of ascorbic acid. Repeated assays on the same urine extracts with the same alkali revealed that the age of a well stabilized reagent does not affect urinary 17-ketosteroid assays. Hence, colorimetric determinations with the modified reagent are at least as precise as with unstabilized alkali, and the same alkali may safely be used for long periods of time.

EXPERIMENTAL

Colorimetric Technique.—Reaction mixtures were prepared as specified by Callow et al. (1938). Color development proceeded for 50

minutes at 25°±0.1°, and tubes were then diluted with 10 ml. 70% alcohol and read on an Evelyn photoelectric colorimeter with the #520 and #420 filters. Absorption figures refer to L values at 520 mμ where L is 2−log galvanometer reading. All determinations were made in triplicate. Volumes of samples were chosen to contain 25 to 35 μg. 17-ketosteroid equivalent whenever feasible in order to minimize a possible deviation from Beer's law, although this is usually negligible with the Callow procedure (Nathanson and Wilson, 1943).

TABLE II. COMPARISON OF VALUES FOR ABSORPTION AND 17-KETOSTEROID CONTENT OBTAINED WITH STABILIZED AND UNSTABILIZED ALKALI ON THE SAME URINE EXTRACTS

	Days Age of Alkali	Blank L	K_D	Type of Urine Extract or Whole Neutral Fraction Number											
				Ketonic*			High Ratio*			Low Ratio*			Low R+20 μg. D*		
				L	Obs. μg.	Fraser μg.	L	Obs. μg.	Fraser μg.	L	Obs. μg.	Fraser μg.	L	Obs. μg.	Fraser μg.
Stabilized	—	.100	9.0*	.254	28.2	26.3	.228	25.4	24.4	.226	25.2	6.8	.372	41.4	25.4
Unstabilized	0	.197	8.7	.255	29.4	27.0	.224	25.5	24.5	.227	26.0	10.4	.375	43.2	27.8
Unstabilized	3	.356	†	.231	26.7	23.8	.202	23.2	21.4	.193	22.2	7.2	.356	41.0	26.4
				♀ Addison's						Non-Ketonic					
				Alone*			+20 μg. D*			Alone*			+20 μg. D*		
Stabilized	—	.104	9.0*	.140	15.6	1.6	.299	33.2	20.0	.150	16.7	5.1	.316	35.2	24.0
Unstabilized	0	.212	8.7	.113	13.0	2.6	.285	32.8	21.1	.132	15.2	7.6	.318	36.6	28.4
Unstabilized	3	.365	†	.081	9.3	—	.259	29.8	17.3	.113	13.0	6.1	.303	34.8	24.8
				2			8			9			10		
Stabilized	—	.112	9.0*	.267	29.4	27.0	.223	24.7	18.4	.218	23.7	15.3	.214	25.2	14.6
Unstabilized	0	.152	9.5	.271	28.6	26.8	.232	24.4	17.9	.221	23.3	15.4	.218	23.0	14.8
Unstabilized	2	.357	†	.234	24.7	24.2	.184	19 4	14.5	.170	17.9	12.3	.125	13.1	9.4

* The values given are averages of the 13 determinations on these same extracts shown in Table I.

† The μg 17-ketosteroid equivalent was calculated using the K_D found at zero days with the same alkali. The K_D values actually observed in each instance were 7.9, 8.2, and 8.5 respectively.

"Fraser μg." is the calculated μg. in the sample used when readings were corrected by the Fraser formula.

The Effect of the Age of Stabilized Alkali on Urinary 17-Ketosteroid Assays

Table I shows the effect of the age of stabilized alkali on the observed total 17-ketosteroid equivalents of urine extracts which differed widely in content of 17-ketosteroids and of interfering chromogens. The K values of dehydroisoandrosterone and the calculated 17-ketosteroid equivalents of the urine extracts remained essentially constant for the 87 days' duration of the experiment. The readings at 420 mμ were also essentially unchanged.

The Effect of the Presence of Ascorbic Acid on the Color Development of Crystalline 17-Ketosteroids

Chromogenic capacities of androsterone, dehydroisoandrosterone, isoandrosterone, etiocholanolone and androstenedione were seen to be essentially the same with stabilized and with fresh unstabilized alkali. Maximum K values obtained with the stabilized reagent were more reproducible, and probably more precise, as they were not subject to errors resulting from deterioration. These determinations have been reported (Wilson and Nathanson 1945).

The Effect of the Presence of Ascorbic Acid on Urinary 17-Ketosteroid Assays

A number of widely differing types of urine extracts were assayed with stabilized alkali at varying intervals after preparation, and also with fresh unstabilized reagents. These included the same extracts described in Table I, and fourteen other whole neutral fractions in addition. Table II shows typical findings. It can be seen that the presence of ascorbic acid did not significantly alter the observed urinary 17-ketosteroid values. The formula of Fraser et al. (1941) was applied to correct for interfering chromogens. The same corrected 17-ketosteroid values were again obtained with both types of alkali.

The Effect of Ascorbic Acid on the Color Development of Crystalline 17-Ketosteroid Added to Urine Extracts

In order to observe the color development of a known amount of 17-ketosteroid in the presence of the interfering chromogens of urine extracts, dehydroisoandrosterone was added to three extracts containing little or no 17-ketosteroids. These were a neutral fraction of low 520/420 ratio, an extract from a woman with Addison's disease, and a non ketonic fraction. The absorption resulting from 17-ketosteroids alone was calculated by means of the Fraser formula before and after the addition of dehydroisoandrosterone. As is shown in Table II, recovery of the added 17-ketosteroid was not appreciably affected by the presence of ascorbic acid.

Errors Resulting from Deterioration of Unstabilized Alkali

Readings obtained with unstabilized alkali after the blank absorption had risen (even though no yellow color had appeared), were subject to greater divergence at 520 mμ than with fresh reagents, and readings at 420 mμ were often more erratic. Moreover, as shown in Table II, all readings at 520 mμ were markedly lowered with such alkali when the blank absorption was elevated. Thus the K value for dehydroisoandrosterone found with fresh unprotected reagent was higher than that obtained with the same alkali two or three days later. Consequently, unless the absorption of standard and extract were measured simultaneously, calculated values with such reagents would be in error. The possible extent of such errors is illustrated in Table II. The higher K values found with fresh alkali are applied to the lowered urine extract readings obtained with the same alkali two or three days later, resulting in lower figures for 17-ketosteroid content. None of these difficulties was encountered with the stabilized potassium hydroxide.

Other Reducing Agents

Hydroquinone and hydrazine sulphate were tried in place of ascorbic acid. Neither can be used in the Zimmermann reaction as the

reaction mixtures turned brown. Hydrazine sulphate was more efficient than ascorbic acid in delaying the appearance of yellow resinous products in a solution of sodium ethylate. Hence it may be useful as a preservative when alcoholic alkali solutions are to be used for other purposes.

SUMMARY

The 2.5 N alcoholic potassium hydroxide used in the Callow modification of the Zimmermann reaction may be preserved for three or more months with ascorbic acid if kept in an atmosphere of nitrogen. Chromogenic capacities of crystalline 17-ketosteroids or those in urine extracts are not altered by either the presence of ascorbic acid or the age of the stabilized reagent.

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THE URETHRAL SMEAR AS AN INDICATOR OF SEX HORMONE IN MALE RABBITS[1]

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ANDREWS (1946) first reported the possibility of the clinical use of the urethral smear as an indicator of androgen levels in men. Cohen (1947), investigating the suggested technic further, concluded that the smear differences which Andrews recorded were due to the drying of some of the slides before fixation, and thus could not be ascribed to different androgen levels. Both Shorr (1941) and Hartman (1944) had reported previously that vaginal smears stained by the Shorr technic require immediate fixation to avoid drying effects.

The work reported herein was carried out on rabbits. We utilized Andrews' (1946) technic as described for man. Preliminary examination of about 20 normal male human urethral smears led us to conclude that the normal cells in man are largely irregularly-shaped epithelial cells with blue-green cytoplasm containing nuclei which may be compact and red-staining or vesicular and blue-to-red staining. We agree with Cohen (1947) that cells with red-staining cytoplasm are either normal epithelial cells which have dried before fixation or cornified cells derived from the junction of the glans and mucosa. However, we find distinct differences in urethral smears of rabbits resulting from experimental alteration of sex hormone states.

METHODS

A total of 41 male Dutch rabbits, six to 17 months of age, was used in this study: 12 untreated controls, seven treated with androgens, 17 treated with estrogens, and five initially untreated castrates.

Androgens employed were testosterone and testosterone propionate.[4] The former was implanted subcutaneously as pellets and maintained in four intact animals for 90 days. The latter in oil was injected intramuscularly into three castrates in daily dosages of 0.25 mg. or 2.5 mg. for 10 to 40 days.

Estrogens employed were estrone, α-estradiol, and estradiol dipropionate.[4] Estrone in oil was injected intramuscularly into six castrate animals in daily dosages of 0.02 mg. or 0.2 mg. for 10 to 40 days. α-Estradiol and estradiol dipropionate were implanted subcutaneously as pellets and maintained in nine castrate and two intact animals for 90 to 400 days.

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Contact smears of the rabbit urethra were made by direct pressure of a glass slide against the mucosa lining the meatal region. In estrogen-treated and in castrate rabbits, there was an appreciable urethral discharge, resulting in thick smears. The smears were taken on two to three successive days in most cases; on only one day in others. A total of 192 smears was studied.

Shorr's (1941) single differential stain was employed in accordance with standard procedure. The importance of fixation in alcohol-ether immediately after taking the smear cannot be over-emphasized.

RESULTS

The smears obtained from rabbits under different hormonal treatments revealed at least three distinct cellular elements present in varying numbers: (1) normal epithelial cells (E) with light blue-staining cytoplasm, polygonal shapes and small, generally bright red nuclei (Figs. 1 and 5); (2) round to elliptical epithelial cells (R), with light to dark blue-green-staining cytoplasm, often vacuolated, and with large, vesicular, red to blue-green nuclei (Figs. 3 and 4); (3) leucocytes (L),

TABLE I. OCCURRENCE OF CELL TYPES IN RABBITS UNDER DIFFERENT SEX HORMONAL CONDITIONS

Animal group	Cell types			Urethral discharge
	Normal epithelial E	Round epithelial R	Leucocyte L	
Normal Adult	+	−	±	−
Castrate	+	+	+	+
Androgenized				
Castrate	+	−	−	−
Intact	+	−	−	−
Estrogenized				
Castrate	±	+	+	+
Intact	−	+	+	+

+ constant occurrence in large amounts.
− absent or infrequent.
± occasional occurrence in moderate amounts.

generally polymorphonuclear (Figs. 3, 4, and 7). There were also many epithelial cells seemingly transitional between the normal E cell and the R cell. We consider the latter to be a younger cell on the basis of its rounded form and its staining reaction. Shorr (1941) reported that younger, non-cornified, vaginal cells stained a deeper (blue-)green than older ones. In addition to the above three types, almost all smears showed varying numbers of reddish-staining, non-nucleated, cornified cells, frequently in large clumps. Cohen (1947) pointed out that similar cells in man probably are derived from the region of the muco-cutaneous junction.

The relative incidence of the three urethral elements described above was recorded for all smears. Table I shows the dominant cell

types characterizing smears from the different groups of animals and summarizes the descriptions given below.

Smears from normal mature rabbits showed almost exclusively type E cells, although occasional leucocytes were present and, rarely, a few cells of type R (Figs. 1 and 5).

TABLE II. URETHRAL SMEARS FROM RABBITS CASTRATED, SOME WITH ANDROGEN REPLACEMENT

Animal number	Pre-castration smears	Operation	Days post-castration										
			7	14	21	28	35	42	49	Operation	56	63	70
1	—	Bilateral cast.	—	ERL	—	—	—	—	—	—	—	—	—
2	E	Bilateral cast.	—	(E)RL	E(R)	ERL	ERL	ERL	ERL	Androgen implanted	ELi*	E	E(i)
3	E	Bilateral cast.	E(L)	E(R)L	ER	ERL	ERL	RL	ERL	Androgen implanted	EL	E(R)	E
4	E(L)	Bilateral cast.	E(R)	ERL	ERL	ERL	ERL	ERL	(E)RL	Androgen implanted	ERL	E	E
5	E(L)	Unilateral cast.; one testis cryptorchid	E	E	—	E	E	E	E	Cryptorchid testis removed	E	ERL	(E)RL

Parentheses indicate small numbers of cell type.
* i indicates very small round cells, not type R.

Smears from intact or castrate rabbits treated with androgens showed a smear similar to that of normal untreated rabbits: almost entirely type E cells (Fig. 2). No observable differences were detected with the several dosages used.

Smears from intact or castrate rabbits treated with estrogens showed relatively few cells of type E, large numbers of type R, and numerous leucocytes (Figs. 3 and 4). There was a copious urethral discharge in almost every animal. The smears with all dosages were indistinguishable.

Following castration, smears retained the characteristics of those of normal animals for approximately one week. Later, they assumed

DESCRIPTION OF FIGURES

All photographs are from smears stained with Shorr's single differential stain.

FIG. 1. Urethral smear from normal control. Large numbers of polygonal epithelial cells (E) with rare round cell (R) and leucocyte (L). 350×

FIG. 2. Urethral smear from intact rabbit with testosterone pellet implanted and maintained for 90 days. Same as Fig. 1. 350×

FIG. 3. Urethral smear from castrate rabbit with estradiol dipropionate pellets implanted and maintained for 90 days. Round cells with vacuolated cytoplasm and vesicular nuclei; numerous leucocytes. 350×

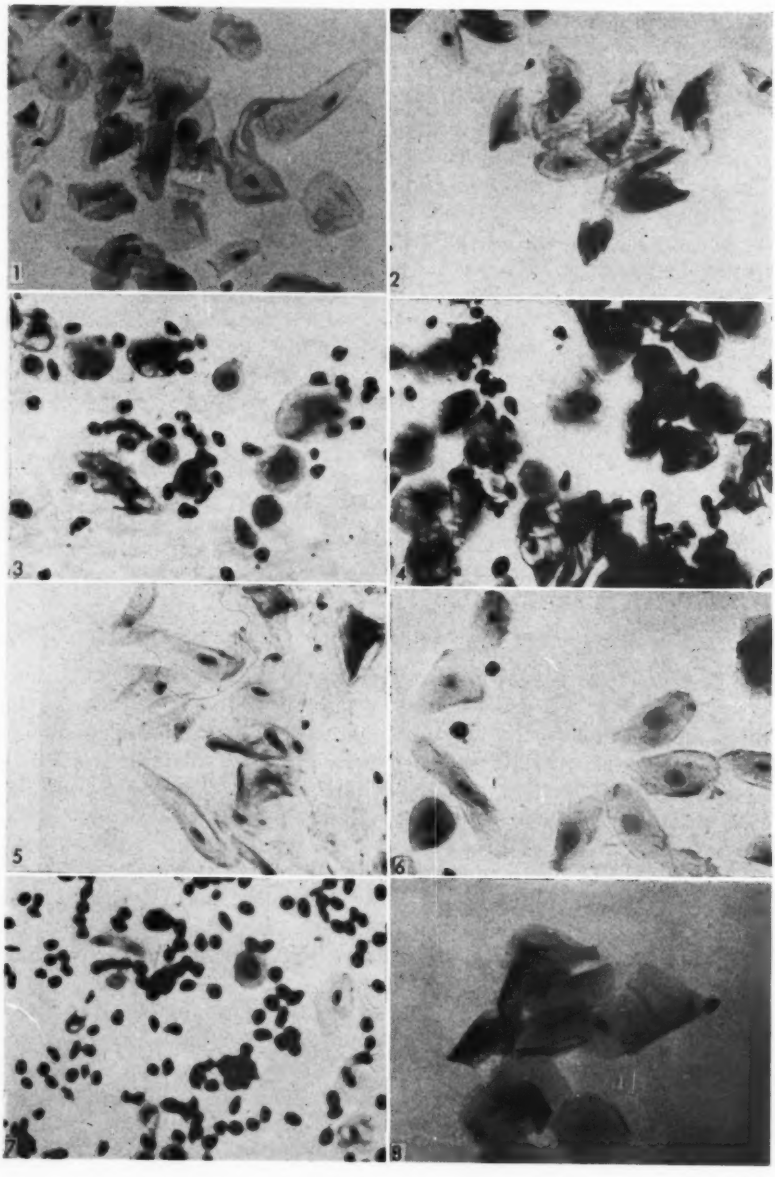
FIG. 4. Urethral smear from castrate rabbit with estradiol dipropionate and α-estradiol pellets implanted and maintained for 210 days. Large numbers of R cells and leucocytes. 350×

FIG. 5. Urethral smear from normal animal, number 4, Table II. Normal smear. 350×

FIG. 6. Urethral smear from number 4, one week after castration. Some polygonal cells with large R-type nuclei; occasional leucocyte. 350×

FIG. 7. Urethral smear from number 4, four weeks after castration. Some R and E cells; mostly leucocytes. 350×

FIG. 8. Urethral smear from number 4, three weeks after implantation of testosterone pellet. Normal picture; compare with Fig. 5. 350×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characteristics similar to those of estrogenized animals with large numbers of type R cells, leucocytes, and a few E cells (Fig. 7). Again, the urethral discharge was copious.

Table II shows the results of castration on the urethral smear and the role of androgen in maintaining and restoring the normal picture. Four animals were bilaterlly castrated, and smears were taken on three of these weekly for seven successive weeks. At the end of this time, androgen (testosterone) pellets were implanted subcutaneously on the medial surface of the right thigh (animals 2, 3 and 4), and urethral smears were read for the three weeks following. By the second week, the normal (pre-castration) picture returned. Figures 5 to 8 show successive smears on one animal (number 4, Table II) to illustrate these changes. The normal picture (Fig. 5) is only slightly altered one week after castration (Fig. 6) with the appearance of large R-type nuclei in some E-type cells. Four weeks after castration, the smear cannot be distinguished from that resulting from estrogenization (Fig. 7). Three weeks after androgen administration, the normal smear is again obtained (Fig. 8).

In one animal (number 5, Table II), only one testis was removed initially, the other remaining cryptorchid. The normal smear was obtained until after the removal of the second testis, at which time the castrate smear resulted.

DISCUSSION

The results of these studies show that there are significant differences in the urethral smears of adult male rabbits under different sex hormone treatments. Estrogenized animals show an abundance of R cells which tend to replace the E cells found in untreated animals. Leucocytes in abundance also characterize all estrogen-treated rabbits, whereas only a few leucocytes are found in normal untreated rabbits. A similar smear is obtained in all castrate animals. Thus, in both estrogenized and castrate animals the urethral smears are characteristically and constantly different from the normal due to the predominance of R cells and leucocytes. Androgenized animals, on the other hand, show smears indistinguishable from the normal consisting almost entirely of E cells.

(It is interesting to note that two out of six untreated older males (over 2½ years) examined gave smears approaching those of estrogenized rabbits.)

The urethral smear of the male rabbit, then, is an indicator of sex hormonal states. This is not surprising, since Russell (1947) has pointed out that the mucosal linings of the several body orifices in man react to sex hormone changes. Our data from rabbits do not agree with Andrews' (1946) from man as to the nature of the smear differences supposedly characterizing androgen deficiency or sufficiency. We believe, as does Cohen (1947), that the normal urethral cell is not

the "cornified" red-staining cell described by Andrews, but is the blue-staining cell described by the latter as indicating androgen deficiency. On the other hand, we find definite changes to occur in the urethral smears of rabbits with sex hormonal alterations, although Cohen (1947) does not describe changes in male patients supposedly androgen deficient or undergoing estrogen therapy. It is open to question as to whether the results obtained after estrogenization are due merely to a deficiency in androgen (primary in castrates and secondary in intacts) or the results after castration are due to the influence of endogenous estrogen.

SUMMARY

A study of urethral smears from male Dutch rabbits indicates that there are distinct and constant changes from the normal smear in castrate and estrogenized animals.

The Shorr technique was employed throughout, immediate fixation being essential to bring out these changes. In normal and androgenized rabbits, urethral smears show a predominance of irregularly-shaped epithelial cells with light blue-staining cytoplasm and small red nuclei. In estrogenized and castrate rabbits, on the other hand, rounded epithelial cells with light to dark blue-green staining cytoplasm, often vacuolated, and with large, vesicular, red- to blue-green nuclei predominate, along with numerous leucocytes. Androgen is able to restore the normal smear in castrates.

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SEX AND DIABETES

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STATISTICS show that human diabetes does not appear with the same frequency in both sexes, particularly after the age of forty years (Joslin, 1946; Marks, 1947; Dahlberg, 1947; Penrose and Watson, 1945; Vartianen and Vartianen, 1944, etc.)

No sexual differences have been reported in experimental diabetes except by Foglia (1945) in the rat. Probably experimental diabetes is produced too suddenly and with a too great intensity, so that sexual influences have no time to act.

Shapiro and Pincus (1936) technique for extirpation of 95% of the pancreas in the rat has made it possible to produce an experimental diabetes in this species. The diabetes observed in the rat has many advantages from the experimental point of view over that provoked in the dog: a) it is possible to work with a standard animal, because uniform genetic strains can be obtained by inbreeding; b) the ease and low price of breeding these animals allows the use of great numbers; c) they are omnivorous like man; d) they have a short life span, etc.

EXPERIMENTAL

The technique of pancreatectomy developed in this laboratory has already been published (Foglia, 1944). Pancreatectomy takes 5 minutes and only a small portion of the pancreas remains between the common bile duct and the duodenum. This portion is about 5% of the whole gland. We have carefully observed and described the diabetes following this operation.

The operated rats do not become diabetic immediately after the operation. Diabetes appears gradually after several weeks or months with all its classic symptoms and can be described as presenting three phases: 1) *pre-diabetes;* 2) *incipient diabetes;* and 3) *confirmed diabetes.* The last phase is very similar to human diabetes: both are of a mild, prolonged type and can be controlled by diet and insulin (Foglia, 1944).

Sexual difference

In our first series of subtotal pancreatectomized rats it was observed that while males became severely diabetic and died, females became diabetic less frequently and survived in greater number.

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[1] A male rat of 170 g. body weight eat daily 27 g. of a standard diet; a female rat of the same weight eat only 21 g. of the same standard diet.

When pancreatectomy was carried out by the same operator, in normal male and female rats of the same age, and received the same diet, a sexual difference was evident. Blood sugar was estimated every month during six months. An animal was considered diabetic when the blood sugar was found to be over 150 mg.% after 7 hours fasting. The series of males and females were operated and followed at the same time to assure the uniformity of the experimental conditions for both sexes. Table 1 shows that the frequency of diabetes is much higher in males than in females.

TABLE 1. SEXUAL DIFFERENCE IN RATS AFTER 95% PANCREATECTOMY INCIDENCE OF DIABETES 6 MONTHS AFTER OPERATION

	Number of rats operated	Number of diabetics	Percentage
Male rats	93	86	92%
Female rats	120	34	28%

A more detailed study (Fig. 1) shows that the number of diabetic females increases slowly in a straight line. In males there is an abrupt increase during the 2nd and 3rd months and the maximum is reached at the 6th month (Fig. 1).

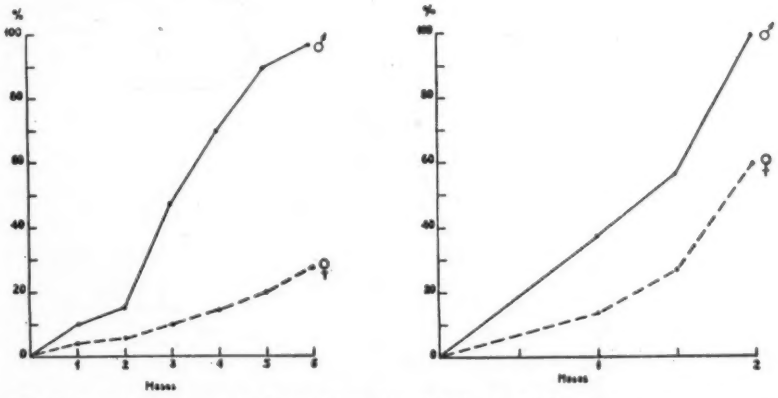

FIG. 1. Sexual difference in the incidence of diabetes in 95% pancreatectomized rats. Left: with ad libitum feeding (93 ♂ and 120 ♀). Right: paired feeding (16 pairs).

Sexual difference and diet

Male rats eat more than females of the same weight.[1] Over-feeding produces diabetes and underfeeding has a protective effect (Martinez, 1946). Therefore the influence of restricted feeding on the appearance of diabetes in males was investigated. Rats of both sexes and the same weight were studied in pairs: the food ingested by the female

was weighed and the same amount was then given to the male. Sixteen such paired-feeding experiments in pancreatectomized animals were made and the results are shown in Table 2 and Fig. 1. Even under these conditions the incidence of diabetes was higher in males.

These results seem to exclude an alimentary factor as the exclusive or principal cause of the sexual difference. Nevertheless these experiments will be repeated giving measured amounts of food by forced feeding.

Sexual difference and gonads

After discarding alimentary factors as the sole cause of the sexual difference the influence of the gonads was studied. The testes might

TABLE 2. MALE AND FEMALE 95% PANCREATECTOMIZED RATS WITH PAIRED FEEDING. THE NUMBERS REPRESENT AVERAGE FIGURES (15 FEMALES, 16 MALES)

Months	Body weight g.		Average fasting blood sugar of diabetics mg. %		Incidence of diabetes %	
	♀	♂	♀	♂	♀	♂
0	89	88	104	108	0	0
1	148	157	154	157	13	38
1½	—	—	171	180	27	56
2	178	190	177	200	60	100
2½	—	—	180	187	—	—

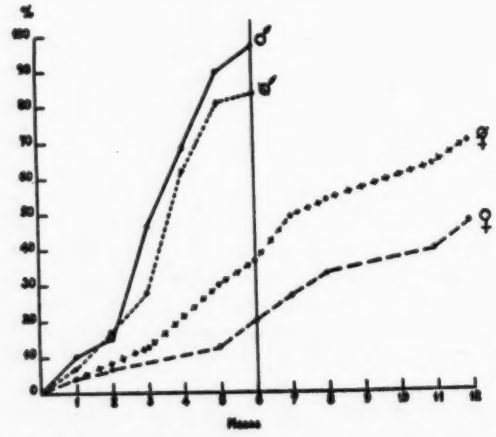

FIG. 2. Influence of castration on the incidence of diabetes after 95% pancreatectomy. Number of rats: 66 ♂; 32 ♂; 30 ♀; 35 ♀.

sensitize to diabetes or the ovaries might have a protective action.

A) *Testes and male hormone.* The role of the testes was first studied

in collaboration with Souto Maior (1944). Simultaneous castration reduces slightly the incidence of diabetes by pancreatectomy. If castration is effected after the onset of diabetes then it produces no difference.

The injection of testosterone (1–5 mg. daily during 10 days) in the prediabetic or diabetic phase produced no effect. In pancreato-adrenalectomized rats, which are very sensitive to diabetogenic influences, testosterone did not produce any effect. As the testosterone treatment given for a short time was ineffective, experiments are being carried out prolonging the treatment during 6 months.

Summarizing our results it can be stated than in the experimental conditions selected only a slight preventive action of castration could be detected.

B) *Ovaries and female hormones.* Female rats were castrated and 8 to 10 days later 95% pancreatectomy was performed. The number of diabetes was higher than after pancreatectomy alone. The difference increased one month after the operation and was important after one year (Fig. 2).

As shown in Fig. 2 the curves corresponding to castrated males and females tend to approximate each other but do not coincide. There are probably other extragonadal factors which maintain the difference between the two sexes even after castration. The greater sensitivity to diabetes of castrated females might be due to the lack of feminine hormones. To test this suggestion estradiol benzoate (2, 4 and 15 μg. daily during six months) was injected to 95% pancreatectomized and castrated-pancreatectomized females. Non injected controls were prepared for each series. Some of the series with the dose of 2 μg. were injected during one year. The results in Table 3 and Fig. 3 show that estradiol exerts a protective effect against diabetes in castrated and whole female rats. Similar experiments were carried out with males (castrated and whole) and here also estradiol reduced the incidence of diabetes (Table 3, Fig. 3).

To summarize: Estradiol benzoate has a powerful protective action against diabetes provoked by 95% pancreatectomy in male and female rats, castrated or whole. The animals injected with larger doses of estradiol (15 μg. daily increased less in weight than the controls and showed pituitary adenomatae. These changes were not observed in the animals receiving smaller doses (2 μg. daily).

The lack of correlation between the dose of estradiol and the frequency of diabetes is probably due to the fact that operations in different series were carried out by different operators and therefore the amount of pancreas left may have varied. In each case the controls of each series were operated by the same person.

The Langerhans islets were studied microscopically. Diabetic rats showed a decrease in β cells; these cells had lost their granulations and some had microvacuolization. This was observed in all the

diabetic rats whether or not they had been treated with estradiol benzoate. Those in which the treatment had a protective effect showed the β cells in good conditions with normal granulations.

TABLE 3. INCIDENCE OF DIABETES IN 95% PANCREATOMIZED RATS, INJECTED DAILY WITH ESTRADIOL BENZOATE DURING 6 MONTHS

Observation during 6 months	Females				Males			
	♀	estradiol ♀	♀	estradiol ♀	♂	estradiol ♂	♂	estradiol ♂
2 μg. daily	6/14	1/12	2/9	2/12	11/12	4/11	8/13	4/11
4 μg. daily	13/63	—	19/52	1/13	8/9	4/8	7/8	4/9
15 μg. daily	5/7	2/6	6/7	1/8	10/10	2/12	8/8	6/9
Observation during one year 2 μg. daily	8/14	1/12	4/9	—	12/12	9/12	11/13	7/11

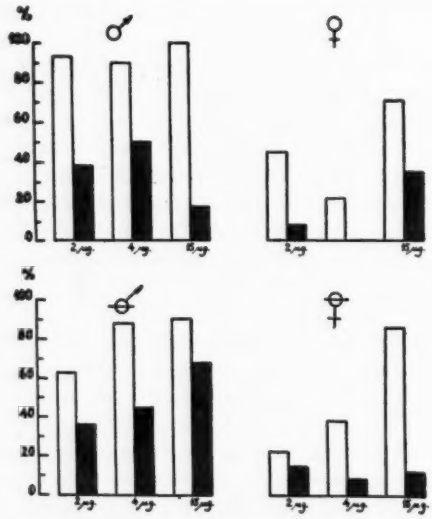

FIG. 3. Influence of injection of estradiol benzoate on the incidence of diabetes after 95% pancreatectomy. Black columns: injected; White columns: controls.

DISCUSSION

The severe diabetes produced by pancreatectomy in dogs led to important progress in the study of this disease. Pancreatectomy in the rat will likewise prove to be an invaluable tool in these studies. The symptoms of these rats appear in three stages: 1) *prediabetes* where the animals are apparently normal offers an animal in which the factors which prevent or provoke diabetes can be studied. (An experimental tool which had not yet been at our disposal.); 2) the stage of

incipient of diabetes; and 3) the *diabetic* stage with symptoms and an evolution more similar to human diabetes than other experimental types of diabetes.

Experimental facts prove that males are much more sensitive than females to diabetes due to subtotal pancreatectomy. It seems alimentary factors can be excluded as the sole cause.

This difference between sexes is influenced by the endocrine secretion of the gonads although other factors seem to play a part since castration does not equalize the frequency of diabetes in both sexes. The testes do not seem to have an important role because castration in males only increases slightly the resistance to diabetes. A more fundamental role is played by the ovaries as proved by the fact that their extirpation sensitizes to diabetes and the injection of estradiol has a protective action in both sexes. The protective action of estradiol was produced without changes in body weight or of visible alterations in the pituitary.

It should be noted that estradiol has been studied as a preventive and not as a curative agent.

The action of estradiol may be due to: 1) inhibition of pituitary secretion; 2) action on the adrenal cortex; 3) a peripheric metabolic action on liver, muscle or other tissues; 4) a stimulating action on the islets. These problems will be the subject of a special study.

The well-known increase in the incidence of diabetes in women during the menopause might be explained by the decrease in estrogens or what seems less likely by the increase in pituitary gonadotrophin which would increase the severity of diabetes.

SUMMARY

A study in rats with 95% pancreatectomy has shown that:

Diabetes is more frequent and appears earlier in males than in females.

This difference is not due exclusively to the amount of food ingested.

Castration has a slight protective action in males and sensitizes females to diabetes.

Estradiol has a protective action in both sexes.

There is degranulation of β cells of the islets in rats with diabetes; these cells are normal in animals protected by estradiol.

To sum up the sexual difference seems to be related to the protective action of feminine hormones.

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NOTES AND COMMENTS

COMPLEMENT IN ALLOXAN DIABETES

IT IS WELL KNOWN that the diminution of resistance towards infection in diabetes mellitus has been attributed to multiple factors: Marble, White and Fernald (1938) consider, among others, the following ones as possible: 1) Increase in the contents of glucose in the blood and the tissues. 2.) Subnormal activity of complement. Horster (1934) has found in pancreatectomized dogs and Richardson (1933) in human diabetes that the activity of complement does not differ in these cases from the normal ones or controls, Nevertheless, Bayer and Form (1926) confirmed that the extirpation of the pancreas produces a diminution of complement, which may temporarily be restored by the treatment with insulin and glucose.

MATERIAL AND METHODS

As in other experiments (5, 6, 7, 8, 9,) we have employed dogs of medium weight (8–12 kg.). The diabetes was obtained by injection of 70–80 mg. alloxan per kg. of body weight. Prior to and after the injection we have made determinations of the activity of complement following the technique suggested by Ecker (1946), and glucemia by the method of Folin. After the injection of alloxan, the determinations were made at different times.

RESULTS

TABLE I

Dog No.	Blood sugar	Complement u	Alloxan mg./Kg.	Blood sugar mg.% hours					Complement hrs. Units per cc.					Remarks
				1	2	3	12	24	1	2	3	12	24	
800	60 mg.%	16. 6	80	78	167	—	150	290	16. 6	16. 6	16. 6	16. 6	14	0 at 12 hr.
801	79 mg.%	7. 5	85	99	142	153	120	95	7. 5	7. 5	7. 5	3. 9	3. 9	−52% at 12 hr.
802	100 mg.%	25. 0	80				81	45				16. 6	7. 6	−33% at 12 hr.
804	79 mg.%	16. 6	80				53	210				16. 6	7. 6	0 at 12 hr.
805	90 mg.%	14. 2	80				90	76				7. 6	7. 6	−49% at 12 hr.
806	87 mg.%	16. 6	80				57	90				14. 2	14. 2	−15% at 12 hr.
797	90 mg.%	25. 0	70					185					14. 0	−44% at 12 hr.
798	85 mg.%	20. 0	80					125					14. 0	−30% at 12 hr.
799	70 mg.%	16. 6	80					135					5. 2	−68% at 12 hr.

As may be observed in Table I (with the exception of No. 800–804) in 7 animals of 9 investigated, a diminution of the complement is observed in quantities which vary between 12% and 68% at 12 hours.

It seems to us interesting to observe the lack of correlation between the diabetogenic action of alloxan and the one which the latter exercises directly or indirectly upon complement. This action appears already after 12 hours, while in the majority of the cases the glucemia rises after 24 hours or later.

The diminution of the activity of complement of the blood caused by the injection of alloxan is a fact, we believe, that deserves certain attention, not only because it is data which contributes to the explanation of the diminution of resistance towards infection in diabetes, but because it may also be interesting from the point of view of the biochemical constitution of complement. We interpret, at least provisionally, this diminution of the activity separate

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from the glucemic elevation, as a cause of the perturbations which are produced in the plasma proteins in diabetes, as has been established by Lewis, Schneider and McCullagh (1944); Lewis Moses and Schneider (1947) in man and in the rabbit, and by one of us (Moore and Candela) in the dog.

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